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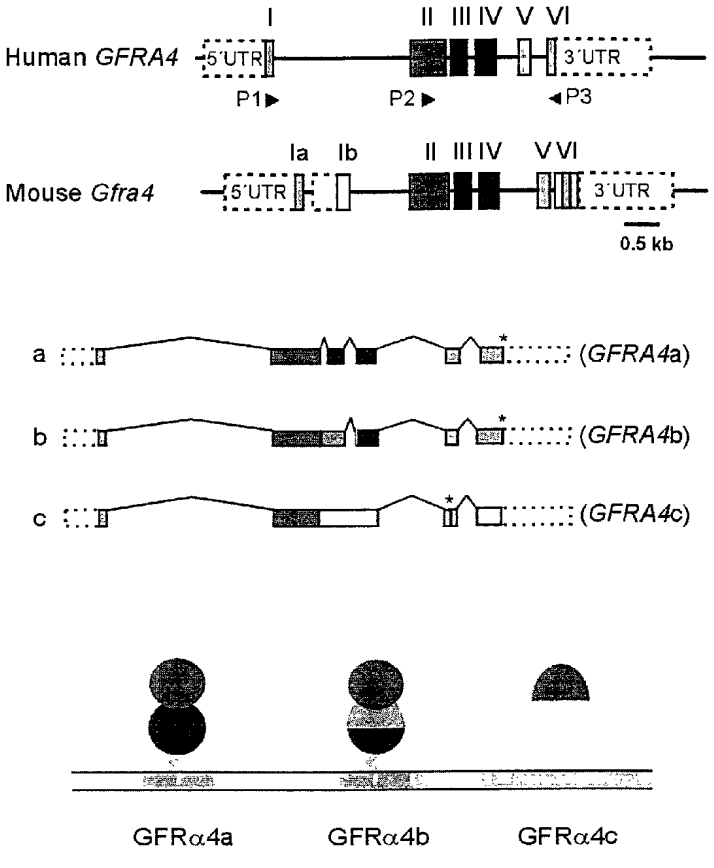

(57) Abstract: The present invention discloses purified and isolated nucleic acid sequences encoding polypeptides having a structure substantially similar to that of splicing forms of mammalian GFRα4 comprising the amino acid sequence (SEQ ID NO:1:) - (SEQ ID NO:6:). The preferred sequences comprise cDNAs having the sequence (SEQ ID NO:7:) - (SEQ ID NO :13:). The present invention is also related to purified and isolated polypeptides comprising the amino acid sequence and or substantially similar splicing forms of mammalian GFRà4. Furthermore, the invention is related to substances capable of specifically recognizing said polypeptides and including both antibodies and receptors. The active compounds of the present invention including cDNAS, polypeptides, binding substances, and antibodies. The invention is useful not only for producing cell-lines and/or transgenic non-human animal but also for diagnosing and treating neuronal disorders or endocrine tumours as well as other related diseases by enabling measurements of GFRα4-mediated signalling in endocrine cells and neurons.

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COMPOUNDS RELATED TO OR DERIVED FROM GFRα4 AND THEIR USE

The Technical Field of the Invention

The present invention discloses compounds related to, derived from or interacting with mammalian GFRα4, a glycosyl-phosphatidylinositol (GPI)-linked GDNF family α-receptor. The invention relates to the mammalian protein as such or derivatives (homologs) thereof, nucleic acid sequences encoding said protein or derivatives thereof as well as modulators or specific binding substances of said GFRα4 or GFRα4-related substances. The compounds are useful especially for diagnosing and treating neuronal or neurological disorders, endocrine cell related diseases as well as tumours by recording GFRα4-mediated signalling on said cells. The GFRα4 or GFRα4-like molecules or compounds interacting with said compounds are also useful for screening mimetics potentially useful as drugs.

The Background of the Invention

The GDNF family of neurotrophic factors includes four members: glial cell line-derived neurotrophic factor (GDNF), neurturin, artemin, and persephin (PSPN). GDNF family ligands signal through receptors consisting of a GPI-linked GFRα subunit and the transmembrane receptor tyrosine kinase RET. In order to activate the transmembrane receptor tyrosine kinase *Ret*, each of the GDNF family neurotrophic factors binds preferentially to one of the glycosyl-phosphatidylinositol (GPI)-linked GDNF family α-receptors (GFRα1-4) (Airaksinen et al., 1999). GFRα4, the preferential receptor for persephin (PSPN), has so far been described only from chicken (Enokido et al., 1998; Thompson et al., 1998). GDNF signals via GFRα1, neurturin via GFRα2, artemin via GFRα3, whereas the mammalian GFRα receptor for persephin (PSPN) and the biological role of GFRα4 has so far remained unclear.

RET-mutations are known to be related with inherited cancer

syndrome (MEN2), characterized by medullary thyroid carcinoma (MTC) and with sporadic forms of MTC and pheochromocytoma, but it is not clear why different specific RET mutations associate with the different disease phenotypes, for example, why hyperparathyroidism and familial MTC associate with certain types of mutations whereas sporadic MTC is associated with other types of mutations. For example *Ret* is expressed in malignant thyroid C-cells and adrenal chromaffin cells but also in normal cells non-affected in MEN2, leaving the cause for the cell specificity of the MEN2 cancer syndrome unclear.

Studies of expression and splicing patterns with mouse and human GFRα4, surprisingly provided a response to the previous unclarities.

The Summary of the Invention

The novel cDNA sequences encoding GFRα4 and corresponding to the mammalian splicing forms of mRNA enable the manufacture of new useful tools for diagnosis and/or treatment of certain endocrine tumours and other diseases related to endocrine cells and/or neurons in mammals, especially humans as well as for identifying and producing new products mimicking the compounds of the present invention.

The present invention is related to an isolated and purified nucleic acid sequence, which is a cDNA encoding a splicing isoform of mammalian GFRα4 having the amino acid sequence (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:), which are mouse derived or (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:), which are human derived. More specifically the cDNA sequences encoding human GFRα4 molecules are characterized by comprising the nucleic acid sequence (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:). The genomic sequence (SEQ ID NO:13:) and fragments thereof including at least one of the exons (SEQ ID NO:29:), (SEQ ID NO:30:), (SEQ ID NO:31:), (SEQ ID NO:33:), (SEQ ID NO:35:), (SEQ ID NO:37:)

or (SEQ ID NO:39:) or any combination thereof with or without introns or parts of the introns (SEQ ID NO:32:), (SEQ ID NO:34:), (SEQ ID NO:36:) or (SEQ ID NO:38:) still encoding a polypeptide having a functional activity substantially similar to that of human GFRα4.

The present invention is also related to the use of said nucleic acid sequence according to claims 1-5 for recording GFRα4-mediated signalling in neurons or endocrine cells.

The present invention is related to the use of nucleic acid sequence according to claim 1-3 for manufacturing polypeptides useful for diagnosing and/or treating tumours in parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma and pheochromocytoma or for treating neuronal or neurological disorders.

The present invention is also related to purified and/or isolated polypeptides having the amino acid sequence (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:), (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) or other peptides, which have a structure substantially similar to an isoform of mouse GFRα4 or of human GFRα4.

The present invention is related to the use of said polypeptides for determining GFRα4-mediated signalling in neurons or endocrine cells and for the use of said polypeptides for treating disorders and disease in the endocrine cells, such as thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells or cells of pituitary intermediate lobe, such as medullary thyroid carcinoma including pheochromocytoma and parathyroid hyperplasia, as well as neuronal disorders.

The present invention aslo include the use of said polypeptides for manufacturing a composition for diagnosing

and/or treating neuronal or neuronic disorders, osteoporosis, cancer in parathyroid gland cells, adrenal chromaffin cells and cells of pituitary intermediate lobe, in neoplasia, including treatment of endocrine tumours, including medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia.

The nucleic acid sequences and polypeptides of the present invention are also useful for recognizing substances which are mimetics of said sequences and are potentially useful as medicines for the diseases mentioned above.

Naturally the present invention is also related to methods for obtaining a cell-line (clone) useful in the production of cells, cell-lines and transgenic non-human animal.

The characteristics of the present invention are as defined in the claims.

Deposition:
The sequences reported in this paper have been deposited in the GenBank database and the accession numbers are listed below. Mus: AJ276870, AJ276871, AJ276872, AJ276514, AJ276515, AJ276516. Human: AJ291673, AJ291674, AJ291675

The Detailed Description of the Invention

Abbreviations
ARTN, artemin; FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; GDNF, glial cell line -derived neurotrophic factor; GFR, GDNF family receptor; MEN, multiple endocrine neoplasia; MTC, medullary thyroid carcinoma; NRTN, neurturin; PSPN, persephin; PTC, papillary thyroid carcinoma, SCG, superior cervical ganglion

Definitions
In the present invention the terms used have the meaning they

generally have in the fields of biochemistry, pharmacology, recombinant DNA technology, including production of transgenic animal but some terms are used with a somewhat deviating or broader meaning than in the normal context. Accordingly, in order to avoid uncertainty caused by terms with unclear meaning some of the terms used in this specification and in the claims are defined in more detail below.

As used herein, **"a neurotrophic factor"** refers to a protein that modulates a biological activity of a cell, particularly neurons, but also other cells, through a neurotrophic factor signalling pathway, such as the RET signalling pathway, serine/threonine kinase Akt-mediated pathway or the RAS/MEK/MAPK pathway. Examples of biological activities in the present invention include, but are not limited to persephin.

As used herein, **"RET signalling pathway"** includes a cell, e.g., neural cell signalling pathway which involves the tyrosine kinase receptor RET. Said pathway includes the GDNF, neurturin, artemin and persephin signalling pathways, which neurotrophic factors bind specific GFRα coreceptors, GFRα1-GFRα4, which in turn bind and activate RET. As used herein "neurotrophic factor signalling pathway" includes a ligand, e.g. persephin (PSPN) independent signalling by GFRα4/RET or GFRα4 without RET.

As used herein, **"a neurotrophic factor responsive cell"** includes a cell which has a biological activity that can be modulated (e.g., stimulated or inhibited) by a neurotrophic factor. Examples of such functions include mobilization of intracellular molecules, which participate in a signal transduction pathway, production or secretion of molecules, alteration in the structure of a cellular component, cell proliferation, cell migration, cell differentiation, and cell survival. Cells responsive to neurotrophic factors preferably express a neurotrophic factor receptor, e.g., a GFRα receptor, such as GFRα4, and/or tyrosine kinase receptor, e.g., the

tyrosine kinase receptor RET. Examples of neurotrophic factor responsive cells of the present invention include neurons, endocrine cells, such as thyroid C-cells and cells in adrenal medulla expressing GFRα4.

Depending on the type of cell, the response elicited by neurotrophic factors is different. For example, in neuronal cells, neurotrophic factors regulate neuron survival and neuronal function. Abnormal or aberrant activity of proteins involved in specific trophic signalling pathways, can lead to a variety of disorders, for example in the endocrine system, e.g., abnormal growth (dysplasia or hyperplasia) as well as abnormal development, differentiation or function of the cells.

Abnormal or aberrant activity of a GFRα4-protein (or abnormal or aberrant nucleic acid expression of the nucleic acid encoding said protein in a neurotrophic signalling factor pathway, in the nervous system or the endocrine system includes the above mentioned disorders in cells expressing or missexpressing GFRα4.

In addition, neurotrophic factors, as such or combined with GDNF, artemin and neurturin and the interaction with GFRα-receptors also promote proper development of a variety of cell types. For example, neurotrophic factor/receptor interactions promote development, differentiation or function of certain peripheral organs and cells of the nervous system, innervation associated with said organs, tissues or cells. Activity of GFRα4 signalling pathway in endocrine systems can lead to disorders including tumours associated with cellular development of cells of these organs as shown in the present RET invention. For example, axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures may aggravate the disease process. Augmenting GFRα4 type of signaling in such conditions could be useful by preventing neuronal death without producing aberrant sprouting.

The term **"GFRα4"** in the present invention means mammalian GFRα4 and/or GFRα4-like or GFRα4-derived protein molecules or polypeptides, i.e. soluble receptor analogues expressed by and being substantially homologous to GFRα4 at amino acid level. Said GFRα4-like or GFRα4-derived molecules defined in the claims are obtainable by isolation from natural mammalian, e.g. human or murine sources as different splicing forms. Said splicing forms lack the first Cys-rich domain characteristic of other GFRα receptors or said domain is easily spliced off during transcription or translation. The GFRα4-like molecules of the present invention can also be produced by synthetic, semisynthetic, enzymatic and other biochemical or chemical methods including recombinant DNA techniques.

The amino acid sequences of the splicing isoforms or variants of mammalian GFRα4 are listed below:

(SEQ ID NO:1:)	murine GPI-anchored isoform a1	Fig 18B;
(SEQ ID NO:2:)	murine putative transmembrane isoform a2	Fig 19B;
(SEQ ID NO:3:)	murine secreted isoform a3/4	Fig 20B;
(SEQ ID N0:4:)	human GPI-anchored isoform a	Fig 21B;
(SEQ ID NO:5:)	human putative GPI-anchored isoform b	Fig 22B;
(SEQ ID NO:6:)	human putative soluble isoform c	Fig 23B.

Polypeptide fragments useful for identifying and isolating nucleotide sequences encoding the above amino acid sequences are (SEQ ID NO:14:), (SEQ ID NO:15:), (SEQ ID NO:16:) and (SEQ ID NO:17:).

The **"GFRα4-like or GFRα4-derived molecules"** are substantially homologous with the amino acid sequences of mouse origin (SEQ ID NO:1:), (SEQ ID NO:2:),(SEQ ID NO:3:) as well as of human origin (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:). They

all lack the first Cys-rich domain characteristic of other GFRα receptors and chicken GFRα4. The "GFRα4-like or GFRα4-derived molecules" are characterized by comprising polypeptides such as the amino acid sequences (SEQ ID NO:14:), (SEQ ID NO:15:), (SEQ ID NO:16:) and/or (SEQ ID NO:17:) or amino acid sequences being truncated at the N-terminal or C-terminal end of the murine or human amino acid sequences (SEQ ID NO:1:),(SEQ ID NO:2:),(SEQ ID NO:3:), (SEQ ID NO:4:), (SEQ ID NO:5:), (SEQ ID NO:6:), but still covering the contagious and/or overlapping regions of the mammalian sequences (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:), (SEQ ID NO:4:),(SEQ ID NO:5:), (SEQ ID NO:6:).

The term **"substantially homologous"** at amino acid level means that the GFRα4-like protein molecules have a significant similarity or identity of at least 80%, preferably 85 %, most preferably more than 90 % with proteins encoded by human genomic sequence GFRα4 (SEQ ID NO:13:)

In the present invention **"homology"** is determined e.g. using BLAST analysis combined with database searches matching the above defined expressed sequence tags (ESTs) with known sequences in the data bases. Nucleotide and amino acid sequence comparisons are also performed with (BestFit), alignment (PileUp), and phylogenetic tree (GrowTree). Also used are as signal sequence and transmembrane hydrophobicity plots (PeptideStructure) with the SeqWeb program package (Genetics Computer Group, Inc.).

The term **"GFRα4-like molecules and derivatives thereof"** comprise polypeptides having the structure, properties and functions characteristic of GFRα4-like molecules. Thus, the term "GFRα4-like molecules and derivatives thereof" includes GFRα4-like molecules, wherein one or more amino acid residues are replaced by another amino acid residue. Also truncated, complexed or chemically substituted, forms of said GFRα4-like molecules are included in the term **"GFRα4-like molecules and**

derivatives thereof". Chemically substituted forms include for example, alkylated, esterified, etherified or amidized forms preferably with a low substitution degree. Especially, useful are substitutions with small molecules, such as methyl or ethyl and a low substitution degree, because said small molecules and/or low substitution degrees, while stabilizing and modifying the GFRα4-molecules of the present invention, are not prone to change or disturb the properties and functions of the GFRα4-like molecules. The truncated, complexed and/or substituted variants of said GFRα4-like polypeptides can be produced by synthetic or semisynthetic methods, including enzymatic and recombinant DNA techniques. The only other prerequisite is that the derivatives still are substantially homologous with and have the properties and/or express the functions of the domains of GFRα4 and can be used for screening compounds mimicking the binding substances of GFRα4.

The isolated GFRα4 molecules should preferably comprise amino acid sequences which are at least about 70-80% or more homologous to the murine and human amino acid sequences defined above and should be characterized by at least one but preferably several of the following activities:

1) it can interact with (e.g. bind to) a neurotrophic factor, e.g. PSPN.
2) it can interact (e.g. bind to) a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET;
3) it can modulate the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET; and
4) it can bind a neurotrophic factor and modulate a response in endocrine cells, e.g., thyroid C-cells

The term **"GFRα4-like molecules"** otherwise covers all possible splice variants of GFRα4 expressed by mammalian tissues possibly excluding certain rat sequences, which are the thyroid, especially thyroid C-cells, and parathyroid glands

and cells in adrenal medulla and the pituitary intermediate lobe, and possibly cells of the nervous system.

As a conclusion **"GFRα4-like molecules"** in its broadest aspect in the present invention, covers not only GFRα4-like molecules derived from nature, including their isoforms of different origin, but also synthetically, semisynthetically, enzymatically produced GFRα4-like molecules including molecules produced by recombinant DNA techniques. Said GFRα4-like molecules can be used either as separate entities or in any combinations.

The term **"isoform"** refers to the different forms of the same protein, which originate from different sources, e.g. different mammalian species, e.g. human and murine sources in the present case. In the present invention the term, thus, includes fragments, complexes and their derivatives. For example, GFRα4-like molecules can be generated by the cleavage of the corresponding preproprotein. Different reactions, including different enzymatic and non-enzymatic reactions, proteolytic and non-proteolytic, are also capable of creating truncated, derivatized or complexed forms of the GFRα-molecules.

Preferably, all **"GFRα4-like molecules and their derivatives"** should be recognizable using binding substances capable of recognizing the native mammalian, including human and murine GFRα4 or GFRα4-like molecules. Such binding substances are for example the neurotrophic factor PSPN and/or fragments thereof and antibodies specifically recognizing GFRα4.

In the present invention the term **"nucleic acid sequence"** means any isolated and purified nucleic acid sequences encoding mammalian GFRα4 and/or GFRα4-like substances or nucleic acid sequences with substantial similarity still encoding GFRα4-like molecules having an amino acid sequence substantially homologous with the murine sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) and/or the human

sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:), lacking the first Cys-rich domain characteristic of other GFRα receptors (GFRα1, GFRα2, GFRα3). Preferably the nucleic acid sequence should encode or express a polypeptide having the characteristics described above and at least one contiguous amino acid sequence (SEQ ID NO:14:), (SEQ ID NO:15:), (SEQ ID NO:16:) and/or (SEQ ID NO:17:).

The nucleic acids sequences of the present invention belong to the functionally active GFRα4-related compounds of the present invention and they can be used as such or they can be introduced into suitable transformation or expression vectors, which in turn can be introduced into suitable host organisms to provide procaryotic, eukaryotic organisms capable of expressing altered levels of GFRα4 as well as transgenic animals, e.g. knockout mice useful as test animals.

The "nucleic acid sequences" are selected from a group consisting of murine and human cDNAs listed below:

(SEQ ID NO:7:)	murine cDNA a1	Fig 18A
(SEQ ID NO:8:)	murine cDNA a2	Fig 19A
(SEQ ID NO:9:)	murine cDNA a3/4	Fig 20A
(SEQ ID NO:10:)	human cDNA (GPIa)	Fig 21A
(SEQ ID NO:11:)	human cDNA (GPIb)	Fig 22A
(SEQ ID NO:12:)	human cDNA c	Fig 23A
(SEQ ID NO:13:)	human genomic	Fig 24

Nucleotide fragments useful as probes or primers in hybridization or PCR-techniques and for identifying and synthetically preparing the human genomic sequence are (SEQ ID NO:18:), (SEQ ID NO:19:), (SEQ ID NO:20:), (SEQ ID NO:21:), (SEQ ID NO:22:), (SEQ ID NO:23:), (SEQ ID NO:24:), (SEQ ID NO:25:), (SEQ ID NO:26:), (SEQ ID NO:27:),(SEQ ID NO:28:), (SEQ ID NO:29:), (SEQ ID NO:30:), (SEQ ID NO:31:), (SEQ ID NO:33:), (SEQ ID NO:35:), (SEQ ID NO:37:), (SEQ ID NO:39:),(SEQ ID NO:40:). The introns of the human genomic

sequence include 5 introns, the first being an unsequenced about 500 bp long sequence situated between the nucleotides 97 and 98 in (SEQ ID NO:13:), (SEQ ID NO:41:), (SEQ ID NO:42:), (SEQ ID NO:43:), (SEQ ID NO:44:), (SEQ ID NO:45:), (SEQ ID NO:46:), (SEQ ID NO:47:), (SEQ ID NO:48:), (SEQ ID NO:49:) and/or (SEQ ID NO:50:). The second intron has the sequence (SEQ ID NO:32:), the third intron has the sequence (SEQ ID NO:34:), the forth intron has the sequence (SEQ ID NO:36:) and the fifth intron has the sequence (SEQ ID NO:38:). It is to be noted that the splicing variants may comprise introns or parts thereof.

The **"nucleic acid sequences"** of the present invention are not in their natural state but are isolated from their natural environment as transiently expressed mRNAs from different mammalian tissue. Thereafter, the mRNAs are purified and multiplied *in vitro* in order to provide by technical means new copies, which are capable of encoding said mammalian GFRα4 or substantially homologous "GFRα4-like molecules" of the present invention. Also the genomic sequence (SEQ ID NO:13:) and its complementary sequence are reprosuced by copying the original strand or overlapping fragments thereof.

The isolated nucleic acid sequences of the present invention also include the human GFRα4 encoding nucleic acid sequences SEQ ID NO:10:, SEQ ID NO:11: or SEQ ID NO:12:, which are a cDNAs identified by aid of the mRNAs from cDNA libraries from human thyroid and adrenal tissues as well as the genomic sequence (SEQ ID NO:13:) encoding and expressing a GFRα4 protein or fragments thereof, which act as a receptor of the corresponding ligand, e.g. persephin.

The term **"genomic sequence"** means the corresponding sequence present in the nucleus of the mammalian cells and comprising introns as well as exons. In the present invention the genomic sequence is included as such and/or as separate exons or introns or combinations thereof. The splicing variants for

example may contain exons and part of introns as shown in the examples.

In the present context the term **"cDNA"** means a DNA sequence obtainable by reversed transcription of mRNA transcribed from the genomic DNA sequence. In the present invention the term cDNA also include the complementary strand of the original cDNA. Thus, nucleotide sequences hybridizing with the cDNA include both sequences substantially identical with the cDNA made as a copy of the respective mRNA but also the complementary strands and/or antisense strands. Genomic sequences copied are also included in the term cDNA.

The term **"nucleic acid sequence encoding or expressing GFRα4-like molecules"** means nucleic acid sequences as well as substantially homologous nucleic acid sequences, which comprise at least one contiguous nucleic acid sequence selected from a group consisting of murine sequences (SEQ ID NO:7:), (SEQ ID NO:8:) or (SEQ ID NO:9:) as well as the human sequences (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:)

Among especially preferred nucleic acid sequences the contiguous sequences (SEQ ID NO:10:), (SEQ ID NO:11:) or (SEQ ID NO:12:) deduced from human cDNA can be mentioned. This sequence or their complementary sequences or nucleic acid sequences comprising parts thereof, e.g. fragments of said nucleotide sequences truncated at the 3'-terminal or 5'-terminal end, as well as sequences containing point mutations or SNPs caused by single nucleotide polymorphism are especially useful as probes for detecting nucleic acid sequences of the present invention.

Specific nucleic acid sequences useful as primers and/or probes are the nucleotide sequences (SEQ ID NO:18:), (SEQ ID NO:19:), (SEQ ID NO:20:), (SEQ ID NO:21:), (SEQ ID NO:22:), (SEQ ID NO:23:), (SEQ ID NO:24:), (SEQ ID NO:25:), (SEQ ID

NO:26:), (SEQ ID NO:27:) and/or (SEQ ID NO:28:), (SEQ ID NO:29:), (SEQ ID NO:30:), (SEQ ID NO:31:), (SEQ ID NO:33:), (SEQ ID NO:35:), (SEQ ID NO:37:), (SEQ ID NO:39:), (SEQ ID NO:40:), (SEQ ID NO:41:), (SEQ ID NO:42:), (SEQ ID NO:43:), (SEQ ID NO:44:), (SEQ ID NO:45:), (SEQ ID NO:46:), (SEQ ID NO:47:), (SEQ ID NO:48:), (SEQ ID NO:49:) or (SEQ ID NO:50:).

It is, however, clear for those skilled in the art that other nucleic acid sequence capable of encoding GFRα4-like molecules and useful for their production can be prepared based on this information. The nucleic acid sequences encoding GFRα4-like molecules should be capable of hybridizing under stringent condition varying e.g between 58°C, 2xSSC and 65°C, 0.1xSSC (Sambrook, J., et al., Molecular Cloning: A Laboratory Manual., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989) with sequence encoding the domain of the GFRα4 or parts thereof.

The nucleic acid sequences of the present invention should have a substantial similarity with the SEQ ID NO:10:, SEQ ID NO:11: or SEQ ID NO:12. **"Substantial similarity"** in this context means that the nucleotide sequences fulfill the prerequisites defined above and have a significant similarity, i.e. a sequence identity of at least 60 %, preferably 70 %, most preferably more than 80 % with the nucleotide sequences encoding the murine amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) as well as the human amino acid sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:).

The term **"nucleic acid sequences encoding human GFRα4 or GFRα4-like molecules"** include their truncated or complexed forms as well as point mutations of said nucleic acid sequences as long as the GFRα4-like molecules are capable of encoding amino acid sequences having the essential structural features as well as the properties and/or functions of GFRα4-like molecules. The functions of the GFRα4 or GFRα4-like molecules or the compounds interacting with said

compounds can be measured with methods generally used for measuring the GDNF family receptor-GFRα mediated signalling including the method disclosed in the example(s) below.

The nucleic acid sequences are useful as such, for preparing transformation vectors and/or expression vectors as well as host cells carrying nucleic acid sequences capable of expressing the desired protein. They are capable of encoding/expressing GFRα4 or GFRα4-like proteins or fragments thereof. The GFRα4 or GFRα4-like molecules or compounds interacting with said molecules are useful for identifying or recognizing compounds mimicking said compounds, respectively. Binding substances are for example antibodies, receptors as well as shorter binding peptides, which specifically bind and recognize GFRα4 or GFRα4-like molecules or compounds interacting with said compounds. Said substances are useful especially for screening mimetics which may be useful in therapeutic or diagnostic applications. Such methods are well known to those skilled in the art. Examples of such methods it is referred to the following patent applications EP 639 584, WO 9638553, US 5,571,506, WO 200018790, WO 9950439, WO 9945930, US 6,030,619 and/or WO 9906599 which by reference are incorparated into the specification of the present invention.

The GFRα4-like molecules can be identified and their amounts can be measured using **"binding substances"**. The term "binding substance" means any substances capable of specifically recognizing and binding the GFRα4-like molecule or derivatives thereof or at least one specific portion of said GFRα4 molecules. Such substances are, ligands of the GFRα4 receptor or other binding proteins or peptides, comprising e.g. specific portions of the GDNF family neurotrophic factor, especially persephin (PSPN), capable of specifically binding GFRα4-like molecules and taking part in the signalling specific for GFRα4. Above all binding substances mean antibodies capable of specifically recognizing one or more GFRα4-like molecules

alone or in any combination. The antibodies include both polyclonal and/or monoclonal antibodies as well as fragments thereof. Preferably, such binding substances recognize and bind to specific epitopes or active sites of the GFRα4-like molecules.

Said binding substances can be produced using specific domains of GFRα4 or any GFRα4-like molecules, their isoforms as well as their fragments, derivatives or complexes with the prerequisite that they are capable of acting as "antigens". The antigens include any compositions or materials capable of eliciting an antibody response specific to said GFRα4 or GFRα4-like molecules or compounds interacting with said molecules. Ligands of GFRα4-receptors, in other words specific neurotrophic factors are also included in the term binding substances. Said binding substances, preferably antibodies can be produced by conventional techniques for producing polyclonal antibodies as well as monoclonal antibodies. The methods for preparing monoclonal antibodies include hybridoma techniques. Fragments of antibodies or other binding proteins such as specific binding peptides can be developed by phage display techniques and produced by recombinant DNA techniques. All methods are well known by those skilled in the art and described in laboratory handbooks.

The term **"screening"** means that arrays of substances produced or recognized e.g. by combinatorial chemistry, with genomics or proteomics can be screened against the GFRα4 compounds of the present invention or their binding substances in order to find compounds mimicking the properties or effects of said GFRα4 or GFRα4-like molecules and/or their binding substances are potentially useful as drugs or as models for developing further compounds. The results can be analyzed by methods provided by efficient tools in modern bioinformatics.

The term **"diagnosing"** means judging, predicting, assessing and/or evaluating as well as identifying and characterizing,

including screening, whether a person is susceptible of or suffers from endocrine cell related diseases or disorders in neurons, e.g. neurite outgrowth leading to neuronal death or aberrant sprouting. The diagnoses also enable evaluation of the severity of the condition, therapy required as well as the efficacy of treatment modalities or medical treatments needed. Especially, early identification of the disease, e.g. neuronal or neurological disorders in order to start prophylactic and/or other treatments before the onset of the actual disease is a desirable feature, enabled by the present invention.

The results are recordable with means for performing immunoassays using GFRα4-like molecules and/or their binding substances as well as parts thereof. Means for performing amplification and hybridization methods using sequence specific probes or primers, which can be selected from the parts of the nucleic acid sequence fragments encoding suitable domains of mammalian, especially human GFRα4.

The term **"screening"** may also mean the screening of a population for the presence or absence e.g. of autoantibodies. This screening means that based on the fact that people or subjects susceptible of or suffering from complications related to a certain said disease caused by GFRα4 or GFRα4-like molecules or compounds interacting with said compounds suffer from aberrant expression patterns which may elicit antibody production not occuring in subjects not suffering from the disorder. These so called autoantibodies can be determined using GFRα4-like molecules. It is easy to screen a large population for the susceptibility of a certain diseases from blood or serum samples obtained from inflowing serum samples taken during routine diagnostic tests. If such autoantibodies can be detected in the serum of a person, it is a clear indication that the person in question is a potential patient and might be susceptible also to endocrine cell-related diseases or neurologic disorders and should be treated appropriately.

The term **"immunoassay"** refers to a immunochemical method or procedure capable of detecting and/or measuring at least one substance, either GFRα4 or GFRα4-like molecules or compounds interacting with said molecules or autoantibodies against said molecules using *per se* known means for performing an immunoassay, which means include a substance capable of specifically recognizing the substance to be determined, i.e. either at least one binding substance or a GFRα4 or GFRα4-like molecules or compounds interacting with said molecules or fragments thereof, for the desired application, respectively.

Well known examples of immunoassays are radioimmunoassays (RIA), radioimmunometric assays (IRMA), fluoroimmunometric assays (IFMA) enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), fluoroimmmunoassays (FIA), luminescence immunoassays, immunoagglutination assays, turbidimetric immunoassays, nephelometric immunoassays, etc. All methods are well known by those skilled in the art and described in laboratory handbooks.

The preparation and development of methods and means for measuring different antigens and antibodies, which can be applied also to the determination of GFRα4-like molecules and their autoantibodies have been described for example in the following patent publications applicable as convenient kits US 5,591,645, US 5,712,170, US 5,602,040, US 5,622,871, US 5,656,503, EP 149 168, US 4,552,839, US 4,361,537, US 4,373,932, WO 86/04683, EP 154 749, EP 7654, WO 86/03839, EP 191 640, EP 212 599, US 4,552,839, EP 158 746, EP 225 054 and which are herewith incorporated by reference. Even if said patents are restricted to the development of test kits for diagnosing other diseases by aid of binding substance recognizing the respective active molecule, the man skilled in the art can use the information for developing corresponding test kits for measuring GFRα4-like molecules of the present invention.

Any immunochemical test methods can in principle be used for diagnosing endocrine diseases as well as for longitudinal or latitudinal screening of the progress of disease and effect of medical treatment. However, visual agglutination, flow-through and immunochromatographic methods are best suited for rapid assays or tests.

The term **"prophylactic treatment"** includes specific measures and/or precautions, e.g.control including possible medication, before the onset of disease. After the on-set of disease, prophylactic treatment requires therapeutic treatment as a precaution in order to avoid further complications.

The term **"therapeutic treatment"** includes methods for treating persons by administrating a compositions comprising GFRα4 or GFRα4-like molecules or compounds interacting with said compounds, including GFRα4-like polypeptides or GFRα4-binding substances. Nucleic acid sequences encoding the same are useful in gene therapy or for preventing the genes causing the disease from expressing the gene products causing the diseases.

The term **"preparation or compositions"** means the active ingredients of the present invention including the nucleic acid molecules encoding GFRα4, GFRα4-like proteins and/or polypeptides, GFRα4 modulators and antiGFRα4 antibodies in combination with at least one pharmaceutically acceptable carrier, which is compatible with the active ingredient, i.e. GFRα4 or GFRα4-like molecules or compounds interacting with said compounds and the route of administration. The nucleic acid molecules of the present invention can be used in gene therapy by introducing the molecules to suitable vectors or other *per se* known delivery systems. The pharmaceutical compositions can be included in a container, pack or dispenser together with instructions for use.

The nucleic acid molecules, proteins, protein homologues, modulators and antibodies described can be used in drug-screening assay, diagnostic assays, methods of treatment, pharmacogenomics, proteonomics, bioinformatics and/or monitoring of effects during clinical trials.

The nucleic acids of the present invention can be used to provide mammalian vectors which in turn can be introduced into mammalian cells. Suitable expression systems for pro- and eukaryotic cells are described in Sambrook et al., 1989. The host cells can be used for expressing the GFRα4-like proteins of the present invention.

The DNA constructs, vector construct and/or host cells of the present invention can also be used to produce non-human transgenic animals useful in screening assays designed to identify agents or compounds, drugs, pharmaceuticals, etc., which are capable of ameliorating detrimental systems related to specific neurons and/or endocrine cells. Especially useful are knockout mice capable of expressing altered levels of GFRα4.

The molecules, including nucleic acid sequences, GFRα4 or GFRα4-like molecules interacting with said compounds and the products developed, characterized and/or produced based on said molecules or compounds of the present invention are useful for studying the role of GFRα4-mediated signalling in neurons, in neoplasia, in endocrine cells and in thyroid calcitonin-producing C-cells (correlated with osteoporosis), parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma, parathyroid hyperplasia and association with specific mutations in the RET oncogene as well as in nervous system and testis.

The General Description of the Invention

The present invention is related to GFRα4 or GFRα4-like molecules or compounds interacting with said molecules which GFRα4-molecules are members of the GDNF protein family including glial-cell derived neurothrophic factor (GDNF), neurturin, artemin and persephin signal through receptors consisting of GPI-linked GFRα subunits and the transmembrane tyrosine kinase *Ret,* but also other signalling pathways.

The present invention is based on preliminary studies made with mouse *Gfra4* which was characterized and shown to undergo developmentally regulated alternative splicing in several tissues. The results also indicated that mammalian GFRα4 receptor lacked the first Cys-rich domain characteristic of the other GFRα receptors.

The glial cell line-derived neurotrophic factor (GDNF) family ligands GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN) are structurally related neurotrophic factors that signal through a multicomponent receptor composed of the transmembrane receptor tyrosine kinase RET and high affinity glycosyl-phosphatidylinositol (GPI)-anchored proteins, the GDNF family alpha receptors 1-4 reviewed by Airaksinen, et al., 1999 and Baloh, et al., 2000. GFRα4 was first described from chicken and shown to be the preferential receptor for PSPN (Enokido, et al., 1998; Thompson, et al., 1998).

The mouse GFRα4 receptor described herein was also disclosed by Lindahl, et al., 2000 after the filing of the priority application. It differs from all other GFRα receptors, including chicken GFRα4 by being smaller in size and lacking the first Cys-rich domain. Mouse Gfra4 transcripts were shown to be expressed in many embryonic and adult tissues but efficient splicing leading to a functional GPI-linked isoform, as well as putative transmembrane and soluble isoforms, occured only in thyroid and adrenal medulla and in pituitary intermedi-

ate lobe. In mouse, Gfra4 and Ret were co-expressed only in the thyroid C-cells and adrenal chromaffin cells. In chicken, Gfra4 mRNA was broadly expressed during embryonic development, including the spinal motoneurons and kidney (Thompson, et al., 1998). Chicken GFRα4 has been shown to bind mouse PSPN and to confer a survival response to PSPN in the presence of Ret (Enokido, et al. (1998). However, due to different structures of chicken and mammalian GFRα4, as well as the lack of information about the existence of chicken GFRα3, ligand-specificity of mammalian GFRα4 cannot be directly extrapolated from experiments with chicken GFRα4.

It is known that PSPN mRNA is expressed at low levels in many rat tissues, where two transcripts, an unspliced and a functional spliced form are produced (Milbrandt, et al., 1998; Jaszai, et al., 1998). PSPN is known to promote the survival of embryonic motor neurons *in vitro* and to rescue nigral dopamine neurons following neurotoxic injury *in vivo* but not to support the survival of any peripheral neurons tested (Milbrandt, et al., 1998). However, unlike GDNF and NRTN, PSPN does not induce neurite outgrowth of rat P8 motor axons in organotypic cultures (Ho, et al., 2000).

Somatic rearrangements were known to cause chromosomal inversions which activate the oncogenic potential of RET in human papillary thyroid carcinomas, whereas germline point mutations are known to be responsible of multiple endocrine neoplasia type 2 (MEN2; reviewed in Edery, et al., 1997; Eng, et al., (1999); Ponder, et al., 1999).

The malignancies associated with these syndromes are known to arise in several neural crest-derived cell populations. MEN2A is characterized by medullary thyroid carcinoma (MTC), pheochromocytomas and parathyroid hyperplasia, whereas MEN2B is characterized by MTC associated with pheochromocytomas, enteric ganglioneuromas, skeletal abnormalities and mucosal neuromas. MTC is the only phenotype in familial medullary

thyroid carcinoma (FMTC). In MEN2A, cysteine substitutions in the extracellular domain of RET cause aberrant homodimerization and ligand-independent constitutive activation of RET (Santoro, et al., 1995; Iwashita, et al., 1996), which alone or together with unknown factors cause the malignant proliferation of cells. FMTC mutations are more evenly distributed among the extracellular cysteines but are also found in the intracellular part of RET. It is known that most cases of MEN2B are caused by a specific mutation in the tyrosine kinase domain, which is also frequently found in sporadic MTC. MEN2B is caused by constitutive activation of RET with altered substrate specificity in downstream signalling pathway (Santoro, et al., 1995); Iwashita, et al., 1996).

However, in MEN2B-RET transfected cells, ligand binding has been reported to increase the intensity of RET signalling (Carlomangno, et al., 1998; Bongarzone, et al., 1998). It has been shown that transgenic mouse models of these mutations cause some of the malignant phenotypes found in human MEN2 syndrome (Michiels, et al., 1997; Smith-Hicks, et al., 2000). Phenotypic variability within the same MEN2 family and between different families carrying the same mutation in RET, suggests that further genetic events or modifier genes are required to induce the tumor phenotype in MTC.

The mouse *Gfra4* cDNA and genomic DNA sequence described in the present invention was characterized by having an unusual structure. The *Gfra4* pre-mRNA was alternatively spliced in tissue-specific manner that was developmentally regulated. A splice variant of *Gfra4* encoding GPI-linked isoform of GFRα4 was selectively expressed in juvenile thyroid C-cells and parathyroid gland. In contrast, newborn and adult thyroid, parathyroid, pituitary and adrenal glands produced another *Gfra4* mRNA species, which encodes a putative transmembrane-anchored isoform. *Ret*-deficient mice were shown to have significantly less C-cells than their wild type

littermates suggesting that thyroid C-cells and possibly adrenal chromaffin cells require GFRα4-*Ret*-signalling, which may specify tissues affected by the MEN2 cancer syndromes.

The nucleic acid sequences of the present invention encode an isoform of mouse or human GFRα4 comprising the murine amino acid sequence (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) as well as the human amino acid sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:). Preferably, the sequences are cDNAs of murine origin having the sequence (SEQ ID NO:7:), (SEQ ID NO:8:) or (SEQ ID NO:9:) as well as the cDNAs of human origin (SEQ ID NO:10:), (SEQ ID NO:11:) or (SEQ ID NO:12:) encoding isoforms of mammalian GFRα4. Also included among the nucleic acid sequences of the present invention are the genomic sequence of human GFRα4 locus (SEQ ID NO:13:).

Originally, a murine GFRα4 nucleic acid sequences was identified by the aid of available genomic sequences in formation of mouse mahogany gene locus (Gunn et al., 1999, Genebank AF155960). Within this genomic region, an "open reading frame" was identified that encoded a protein that showed sequence homology to chicken GFRα4. Probes were generated based on portions of the genomic sequence and cDNA libraries were screened with said probes. Nucleotide sequences were determined and assembled and various methods such as RACE and genomic sequence analysis were used to extend the 5' sequence. Examples of the mammalian nucleotide sequences which were found and which encoded GFRα4 were the mouse cDNAs (SEQ ID NO:7:), (SEQ ID NO:8:), (SEQ ID NO:9:) as well as the human cDNAs (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:) and the predicted amino acid-sequences were the mouse originated sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) as well as the human originated sequences (SEQ ID N0:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) also shown in Figures 18-24.

The present inventors also isolated and characterized the

human GFRα4 receptor and showed that it is a functional co-receptor for PSPN, which mediates RET activation. Selective co-expression of GFRA4 and RET in normal and malignant C-cells and developing adrenal gland suggested that GFRA4 is a candidate modifier gene in medullary thyroid carcinoma and pheochromocytoma associated with the MEN syndromes.

The present invention is based on the amino acid and/or nucleic acid sequence defined above and in the claims, especially, the *GFRα4* cDNA, that encodes a specific member of the Glial Derived Neurotrophic Factor-Alpha Family of Receptors, i.e. the GFRα4 protein, which functions in neurotrophic factor signalling pathways.

The first mammalian members of the *GFRα4* subfamily were identified, as described herein. Gfra4 was expressed in many mouse tissues including nervous system, where intron retention leads to a putative secreted GFRα4 protein. Efficient splicing was shown to occur only in thyroid, parathyroid, pituitary, and less in adrenal glands. A splice form leading to a GPI-linked GFRα4 receptor was shown to be expressed in juvenile thyroid as well as in parathyroid and pituitary glands.

Significant loss of thyroid C-cells in *Ret*-deficient mice suggested that C-cells, and cells in adrenal medulla also express *Ret* and may require signalling via GFRα4-*Ret* receptor. Finally, GFRα4 expression may restrict the inherited cancer syndrome multiple endocrine type 2, associated with mutations in RET, to these cells. These preliminary hypothesis were supported by further observations as disclosed below.

The first GFRα4 proteins isolated were expected to bind to neurotrophic factors, especially GDNF-family ligands, and mediate signals within cells expressing the GFRα4 protein. As expected, it was shown that the GFRα4 protein transmitted a signal to the interior of the cell by activation of the RET

protein tyrosine kinase signalling pathway. Neurotrophic factors are known to promote survival and function of neural cells of both the central and peripheral nervous systems. Thus, modulation of the activity of a molecule involved in transmitting a neurotrophic factor signal to a cell results in modulation of the neurotrophic factor initiated cell function. Consequently, the ability of GFRα4 to modulate neurotrophic factor initiated cell functions were tested. It was shown that GFRα4 can be used to modulate neurotrophic factor action and/or activity and thereby GFRα4 is useful for treating disorders associated with such functions (or lack thereof).

In addition, GFRα4 mRNAs were expressed in a variety of tissues, especially, during embryogenesis, including, for example, cells of the pituitary gland and the intermediate lobe. Thus, modulators of GFRα4 can be used to modulate development of these tissues and thereby to treat disorders associated with abnormal or aberrant development of these various tissues.

The molecular mechanisms by which the mutated Ret alleles contribute to the development of neuroendocrine neoplasms in MEN-2 syndromes is largely unknown. The effects of tyrosine kinase receptors are known to be mediated by the concerted activation of several signalling pathways including those of phospholipase C-, phosphatidylinositol 3-kinase, and the Ras/mitogen-activated protein (MAP) kinase (also known as ERK) (Kazlauskas, et al., 1994). In a rat pheochromocytoma cell line, PC12, activation of the Ras signalling cascade is a prerequisite for nerve growth factor (NGF)-induced cell differentiation (D' Arcangelo, et al., 1993).

Although Ras is implicated in Ret-induced neuronal differentiation, little is known about the contribution of this pathway to the biological effects triggered by Ret mutants in neuroectodermal cells (Rossel et al., 1997). For instance, the expression of active Ret mutants and their

causal function in neuroendocrine tumors, associated with MEN-2 syndromes, are difficult to reconcile with the dramatic differentiating effects observed when the same mutants are overexpressed either in PC12 or in human neuroblastoma cells (Borrello et al., 1995); Califano et al., 1995; Rossel et al., 1997). In PC12 cells, the Ret-induced molecular and morphological changes leading to differentiation are both mediated by Ras-dependent pathways (Califano et al., 2000). The studies of the present inventors using the claimed GFRα4 molecules provided useful new information to this field.

It was generally believed that the serine/threonine kinase Akt-mediated pathway is responsible for most of the neurotrophin-regulated cell survival (for review see Kaplan and Miller, 2000). The evidence for the contribution of Ras/MEK/MAPK pathway to neuronal survival is rather conflicting. While neurotrophins induce a strong and sustained activation of MAPK in sympathetic neurons and PC12 cells, most studies have found that inhibition of MEK has minimal effects on neurotrophin-dependent neuronal survival. The major role for MAPK-induced survival pathways may be to protect neurons from death due to injury or toxicity, rather than from trophic factor withdrawal (Kaplan and Miller, 2000). Recent work of Ashcroft et al., 1999) has shown that while PI-3 kinase can contribute in part to neurite initiation processes, its selective activation and subsequent signalling to downstream effectors such as Akt, functions mainly to promote cell survival in PC12 cells. The Ras/MAPK pathway, on the other hand, is mainly responsible for neurite outgrowth and differentiation (Perron and Bixby, 1999), Ashcroft et al., 1999). Also in this respect the results of the present invention provided new information as regards GFRα4s.

The transforming ability of several viral and cellular oncoproteins depends on their capacity to activate PI3-K.

a) Ret-MEN2A and MEN2B mediate enhanced PI3-K activity and

high phosphorylation state of its downstream signalling molecules;
b) Ret-mediated cell-transforming effect is known to be critically dependent on the activation of PI3-K/Akt pathway (Murakami et al., 1999; Segouffin-Cariou and Billaud 2000). In medullary thyroid carcinoma cell line TT, which carries Ret-MEN2A mutation, introduction of v-Ha-*ras* oncogene or activation of exogenous Raf (a kinase of MEK) leads to TT cell differentiation, a process opposite to oncogenic transformation (Nakagawa, et al., 1987; Carson-Walter, et al., 1998). Also as regards to these articles the present invention provides new useful data.

All members of GDNF family have shown to protect against excitotoxic motor neuron degeneration. In contrast, only GDNF and NRTN but not PSPN are able to induce neurite outgrowth in the organotypic culture of the same motoneuronal population. A specific inhibitor of MAP kinase , PD98059, inhibits the motor axon outgrowth-promoting activity of the GDNF but not the neuroprotective activity. (Ho, et al., 2000). Also as regards neurite outgrowth new data is provided by the present invention.

Because Ret *in vivo* is never expressed alone but always with a GFRα co-receptor (Golden, J. et al., 1999) it has been suggested that there is a difference in the activation of Ret signalling cascades depending on Ret interaction with one of the GFRα co-receptors. The signalling events might be different also when constitutively activated oncogenic Ret acts alone, without a co-receptor. This difference may lead to the predominance of either differentiating or surviving/ proliferating cellular response. The present invention provides information suggesting that the Ras/MAPK pathway, being responsible mostly for cells differentiation in this paradigm, may undermine the action of PI3-K/Akt cascade, which can lead to the oncogenic transformation. The absence or inhibition of Ras/MAPK pathway on top of PI3-K/Akt activation

might be causative in the development of Ret-mediated MEN2 cancer syndrome, which is also shown below in the present invention.

The present inventor showed that unlabelled PSPN displaces [^{125}I] PSPN from GFRA4-transfected Neuro-2a cells, which express endogenous Ret. PSPN can be specifically cross-linked to mammalian GFRα4 and Ret, and is able to promote autophosporylation of Ret in GFRA4-transfected cells. PSPN, but not other GDNF family ligands, promotes the survival of cultured sympathetic neurons microinjected with GFRA4 and RET. Three different splice forms of human GFRA4 mRNA encoding for two GPI-linked and one putative soluble isoform identified by the present inventors were expressed exclusively in the thyroid gland. Overlapping expression of RET and GFRA4 but not other GFRA mRNAs in normal and malignant thyroid medullary cells suggests that GFRα4 may restrict the MEN2 syndrome to these cells.

As already described above the present inventors in their preliminary studies identified from a putative mouse the *Gfra4* gene localized near the mahogany locus (Gunn et al., 1999) putative exons of the *Gfra4* gene homologous with other Gfra receptor genes and identical to several expressed sequence tags (see the Examples). The present inventors used RACE-cloning and RT-PCR of *Gfra4* cDNA from various tissues to identify the 5' and 3' sequences and splice variants of mouse *Gfra4* mRNA. All 5'RACE clones from the thyroid (including parathyroid) glands contained the signal sequence encoded by exon Ia (Fig 1A, 1C).

One 5'RACE clone contained an alternatively spliced exon Ib (Figure 1C), which would be translated into a weak putative signal sequence. All cDNA clones isolated from brain included a 53 bp intron between exons II and III, and the 5' extended up to 100 bp into the first intron upstream of exon II (Figure 1C). Translation of these mRNAs gave rise to a protein without

a signal sequence (not shown)(Figure 18, SEQ ID NO:1: and SEQ ID NO:7:).

The predicted amino-acid sequence of full-length mouse GFRα4 from juvenile thyroid gland consisted of 263 amino-acids with a hydrophobic signal and C-terminal sequences typical for a putative GPI-anchored protein (Udenfriend and Kodukula, 1994). Mouse GFRα4 showed higher amino-acid identity with chicken GFRα4 (53%) than with chicken GFRα1 (40%) and GFRα2 (40%). However, the identity between mouse and chicken GFRα4 was lower than the identity between mouse and chicken GFRα1 (80%) or between the mouse and chicken GFRα2 (75%). The alignment of mouse GFRα1, GFRα2, GFRα3 and GFRα4, as well as chicken GFRα4 is shown Figure 1A.

The sequence of the GPI-anchored isoform of human GFRα4 (Figures 21B and 22B, SEQ ID NO:4: or SEQ ID NO:5:) showed 79% amino-acid identity with the mouse GFRα4 (SEQ ID NO:1:). Phylogenetic comparison indicated that the mouse GFRα4 is more closely related to mouse GFRα1 and GFRα2 than to GFRα3 (Figure 1B). Amino-acid sequence alignment (Figure 1A) showed that mouse GFRα4 protein is smaller than the other GFRα-receptors. The mouse *Gfra4* gene (Figure 1C) contains at least six exons but it lacks the sequences corresponding to exons 2 and 3 of the other *Gfra* genes (Angrist, et al., 1998; Baloh, et al., 1998), which encode for the first Cys-rich domain (D1) and the hinge region in the putative GFRα domain structure (Airaksinen et al., 1999). Thus in contrast to other GFRα-receptors including chicken GFRα4 that have three domains, mouse and human GFRα4 has only two Cys-rich domains (Figure 1D).

Northern blot analysis of *Gfra4* expression in different tissues showed high mRNA levels in the developing and mature nervous system, thyroid gland and testis (Figure 2A). Moderate expression was seen in the adrenal gland and in several embryonic tissues (Figure 2A and not shown). The size of the

major *Gfra4* transcripts in testis (approx. 1.2 kb) shorter than in other tissues, such as 1-week-old brain (approx. 1.4kb). Longer transcripts (approx. 2.5 kb) were also present. (Figure 2A). *Gfra4* mRNA levels were low or not detectable in developing and adult kidney, muscle, spleen, liver, and salivary gland (Figure 2A and not shown).

RT-PCR was used to analyse *Gfra4* expression and to distinguish between the different splice forms in various tissues using primers spanning different exons. *Gfra4* mRNA comprising exon Ia was prominently expressed in the thyroid, parathyroid, and pituitary glands, and less in adrenal gland (Figure. 2B,C). The expression pattern of transcripts containing exon Ib was similar to those containing exon Ia. In testis and brain, in contrast transcripts containing exon Ia or Ib were barely detectable or undetectable, respectively (Figure 2B,C). This suggests that other exons encoding the N-terminal part of GFRα4 protein are used in brain and testis.

Of all tissue examined, only thyroid, parathyroid and pituitary glands expressed major subset of Gfra4 transcripts in which the short 53 bp intron separating exons II and III was correctly spliced (Figure 2B). Splicing of this intron occured also in the adrenal gland but not in the brain or the testis. *Gfra4* transcripts with inclusion of the short 53 bp intron separating exons II and III were present in all tissues examined, including thyroid, pituitary and adrenal glands (Figures 2B and 2C). Translation of this intron causes a frame shift and premature stop codon producing a truncated protein with domain-2 (D2, Figure 1D) followed by a 53 amino-acid hydrophilic tail (SEQ ID NO:15:). This suggests that, regardless of possible signal sequence, testis and brain tissues examined would produce little, if any, membrane anchored GFRα4. These results were confirmed by RNAse protection assay using a probe that spans from exon II to exon IV (see Experimental Methods, data not shown). Translation of the transcripts that contain exon Ia and the intron (a3 and a4

in Figure 2C) would lead to production of a putative truncated isoform of GFRα4 with 190 amino acids and strong signal sequence (SEQ ID NO:3:). In contrast, possible translation products of *Gfra4* transcripts that contain exon Ib and the intron (*b3* and *b4* in Figure 2C) might be poorly secreted.

Another alternative splicing event, which was shown to be developmentally regulated, involved the last exon (exon IV). A splice form that encodes the putative GPI-linked receptor was selectively expressed in the thyroid and parathyroid gland of juvenile 3-week-old animals (Figure 2B, transcripts a1 and b1 in Figure 2C.) Surprisingly, in newborn and 6-week-old mice, the thyroid and parathyroid glands preferably produced a longer splice form of *Gfra4* mRNA with an alternative usage of splice acceptor site in exon IV (Figure 2B, transcript a2 and b2 in Figure 2C). This exon IVb would translate into a putative transmembrane (TM) isoform of GFRα4 (SEQ ID NO:2:) with and over 20 amino-acid hydrophobic strech followed by a nine amino acid hydrophobic tail (SEQ ID NO:16:) instead of a putative GPI-anchor signal sequence (SEQ ID NO:17:) encoded by exon VIa. This TM splice variant was also expressed in adrenal and pituitary glands (Figure 2B). In conclusion, the splicing of Gfra4 gene is both tissue-specific (with splicing of the short intron between exons II and III, and use of the signal sequence exon Ia) and also developmentally regulated (with alternative usage of different 5' splice sites of exon VI).

Splice site sequences of the short 53 bp intron are weak (Figure 3) with sub-optimal 3' polypyrimidine tract of the intron between exons V and VIa is also non-canonical. Weak splice sites are typically found at alternatively spliced sites, for example, in the calcitonin/CGRP gene that produces calcitonin in thyroid C-cells and CGRP in neuronal tissue. According to one model, additional RNA processing factors that help to recognize weak splicing sequences in calcitonin/GCRP gene are present in the C-cells (Lou and Gagel, 1998). It is possible that the tissue-specific and developmentally

regulated splicing of the Gfra4 gene uses these same factors. However, although highly specific alternative splicing factors cannot be ruled out, the evidence found suggested the developmental and tissue specificity may arise from different ratios of general antagonistic and cooperating splicing factors as discussed by Lopez, 1998; Grabowski, 1998.

In situ hybridization was used to study the distribution of *Gfra4* and *Ret* mRNAs in mouse tissues (summarized in Table 1). *Gfra* was highly expressed in the intermediate lobe of developing (E14, E16 and P0) and mature pituitary gland (Figure 4A and Table 1), while *Ret* was expressed in the neurohypophysis and not in the intermediate lobe (Figure 4B). Moderate levels of *Gfra4* transcripts were found in several tissues at different embryonic and postnatal stages examined such as the condensing mesenchyme of developing bones (Figure 4C) and developing nervous system (Table 1). In adult spinal cord, *Gfra4* mRNA was present in most principal neurons including motor neurons (Figure 4D). In adult brain, *Gfra4* was expressed in neurons, with higher levels in the cerebral cortex and hippocampus than in the brainstem and cerebellum (Figure 4E). This expression pattern was strikingly different from that of *Ret* (Figure 4F). Moderate *Gfra4* expression was seen in the gut circular muscle and myenteric ganglia (Figure 4G,H), as well as in other peripheral ganglia, including the sensory doral root and trigeminal as well as superior cervical and sympathetic chain ganglia (Table 1). In testis, Gfra4 mRNA was expressed in seminiferous tubules (Figures 4I and 4J). *Gfra4* and *Ret* were co-expressed in the neural crest-derived ultimobranchial body at E12 (Figures 5A-5C), E14, and also E16, when it starts to fuse with the thyroid diverticulum (Table 1).

In newborn mouse, highest expression of *Gfra4* was detected in distinct cells in thyroid medulla where calcitonin-producing C-cells are located (Figure 5D). Hybridization of adjacent sections with *Ret* probe (Figures 5E and 5F) showed a similar

pattern to *Gfra4*. Thus, the thyroid C-cells, which express *Ret* (Tsuzuki et al., 1995; Belluardo et al., 1999), co-express *Gfra4*. In contrast, other *Gfra* (*Gfra1-2*) mRNAs were not detected in the thyroid C-cells (data not shown) consistent with previous data) (Belluardo, et al., 1999; Golden, et al., 1999).

In postnatal thyroid gland, *Gfra4* (Figure 5G) and *Ret* (Figures 5H and 5I) co-expression continued in the C-cells. *Gfra4* mRNA levels remained abundant at least up to 17 weeks while *Ret* levels appeared to decrease (Table 1). Interestingly, the parathyroid gland, which expressed little if any *Gfra4* at birth (Figure 5D), expressed highly in 3- and 6-week old mice (Figure 5G and Table 1). However, we could not detect *Ret* expression in the parathyroid gland by *in situ* hybridization at any developmental stage examined (Figures 5E and 5I as well as and Table 1).

Gfra4 and *Ret* were also expressed by newborn, postnatal and adult adrenal medulla (Figures 5J-5L and Table 1) at P8 while all cells in the medulla appeared to express *Gfra4* (Figure 5J), only a subpopulation of them, presumably chromaffin cells (Nakamura, et al., 1994; Tsuzuki, et al., 1995), contained *Ret* mRNA (Figure 5L).

The tissue-specific splicing, leading to putative GFRα4 isoforms with either GPI- or transmembrane anchor, and co-expression of *Gfra4* and *Ret* in embryonic and postnatal thyroid C-cells suggested a physiological role for GFRα4 in the development of these tissues. To test this possibility, we studied the number of C-cells in thyroid glands from *Ret*-deficient (-/-) mice. The number of calcitonin-immunoreactive cells in E18 *Ret* -/- mice appeared clearly reduced compared to control (wild-type or heterozygous) littermates (Figures 6A and 6B). Counting of immunoreactive profiles from serial reactions throughout the thyroid gland indicated a significant loss of about 37%

(control 2540±240, *Ret* -/- 1600 ± 100, n=4 littermate pairs, P<0.005 using t-test). Thus, some thyroid C-cells seem to require *Ret*-signalling already during embryonic development. The selective expression of *Gfra4* in these cells suggests that GFRα4 mediates the *Ret*-signalling in C-cell development. Other C-cells may require GFRα4/*Ret*-signalling postnatally, as *Gfra4* and Ret are co-expressed in these cells up to adulthood. The adrenal gland has been reported to be unaffected in newborn *Ret*-deficient mice (Durbec, et al., 1996). However, the expression of *Gfra4* and *Ret* in postnatal adrenal medulla suggests that signalling via GFRα4-*Ret* receptor complex may be important in postnatal development of adrenal medullary cells. Finally, the expression of Gfra4 and *Ret* in chromaffin and C-cells suggests that the putative ligand PSPN should have trophic or other biological effects on these cells.

Efficiently spliced *Gfra4* transcripts in mouse are expressed in thyroid, parathyroid and adrenal glands, that is, in tissues affected in humans with MEN2 syndromes, suggesting a causal link. GFRA4 is expressed and efficiently spliced also in human thyroid gland. In MEN2 cancer cells, deregulated RNA processing (Lou and Gagel, 1998) could lead to altered expression of *Gfra4* mRNA splice forms. GFRα4-receptors may bind and modulate specific *Ret* mutants and could be essential for their oncogenic activity.

In the present invention the structure and expression patterns of human GFRα4 are described as well as the cloning of human GFRα4 cDNAs encoding three different isoforms of human GFRα4, two GPI-linked and one putative soluble isoform, from adult human thyroid gland. The cloning and expression of various mouse GFRα4 cDNAs encoding different protein isoforms have been described above. As in mouse, all human GFRα4 isoforms lack the first Cys-rich domain (D1) common for all known GFRα-receptors, including chicken GFRα4 (Thompson, et al., 1998).

Of 25 different adult and two fetal tissues studied, GFRA4 transcripts were detected only in the adult thyroid gland. However, it cannot be excluded that GFRA4 could be expressed in other tissues (such as pituitary intermediate lobe, which expresses high levels of Gfra4 in mouse) or at earlier developmental stages (such as developing adrenal medulla) not analyzed here. However, this apparently exclusive expression of human GFRA4 in thyroid suggests a more tissue-specific transcriptional regulation and possibly a more restricted role for GFRα4 in human, than in chicken and rodents, where Gfra4 is expressed in many tissues (Thompson, et al., 1998). For example, the lack of GFRA4 in developing and adult human nervous system does not support a physiological role for PSPN as dopamine or motor neuron survival factor as was initially suggested using rat neurons (Milbrandt, et al., 1998).

Consistent with previous results disclosed by Nakamura, et al., 1994, Lorenzo, et al., 1995; Tsuzuki, et al., 1995) RET mRNA was expressed at variable levels in many adult human tissues, including the thyroid and adrenal glands. According to Milbrandt, et al., 1998 functional spliced PSPN mRNA is not present in humans. However, the present inventors found unspliced transcript of PSPN in all human tissues examined, whereas only low levels of spliced mRNA were detected in human adrenal gland, cerebellum, spinal cord and testis. In contrast, similar levels of both PSPN transcripts are present in most tissues in rat (Milbrandt, et al., 1998; Jaszai, et al., 1998). Although the spliced transcript encoding functional PSPN is expressed in the rat thyroid (not shown), its expression in either adult human (Figure 10) or postnatal mouse (not shown) thyroid gland could not be detected. Adrenal gland was the only tissue where the spliced PSPN transcript was detected in all three species analyzed (mouse, rat and human). The source of ligand for GFRα4 in human thyroid remains elusive, although secretion of PSPN (from an unknown source) into the blood circulation can be speculated because

PSPN triggered Ret autophosphorylation in cells was detected only after serum deprivation (Poteriaev, D., unpublished data). It is also possible that functional PSPN is expressed in the thyroid region at earlier developmental stages not analyzed here.

Masure, et al., 2000 have reported a Gfra4 cDNA encoding a different GFRα4 isoform. It was cloned from rat brain and reported as a GPI-linked receptor for PSPN. However, the signal sequence encoded by the rat Gfra4 cDNA in that report is very weak and differs from the GFRα4 signal sequence disclosed in the present invention.

Sequence information from the preliminary research works using mouse Gfra4 cDNA and gene was used to identify human GFRA4 cDNA and genomic clones which is the main subject of the present invention. First, the genomic region covering putative exon 2 to exon 4 was cloned by PCR from human genomic DNA. Next, the structure of GFRA4 cDNAs and gene were characterized by 5' and 3'RACE of adult human thyroid cDNA and PCR of genomic DNA using primers corresponding to different regions of the putative exons of human GFRA4 as disclosed in the examples above. The sequence of human GFRA4 exon 1 encoding the signal sequence showed high homology to the mouse Gfra4 exon 1a. cDNAs encoding proteins with an alternative signal sequence homologous to the 1b signal sequence found in mouse, or putative transmembrane and soluble isoforms found in mouse, were not identified from human thyroid. Instead, three different alternatively spliced GFRA4 cDNAs were identified (GFRA4a, GFRA4b and GFRA4c, (SEQ ID NO:10), (SEQ ID NO:11) (SEQ ID NO:12), Figure 9B):

(i) GFRA4a (810 bp) corresponds to the mouse Gfra4 transcript a1. The predicted protein (GFRα4a, 290 amino acids, Figure 9C) contains a putative N-terminal hydrophobic signal, one N-linked glycosylation site (NVSA) at position 178, and a hydrophobic stretch of amino acids in the C-terminus, preceded

by a hydrophilic linker region, consistent with a GPI-anchor signal sequence (Udenfried, et al., 1995). The amino acid identity between mouse and human GFRα4a is 76%, whereas the identity between human and chicken GFRα4 is 54% (covering amino acids 143-340 in chicken GFRα4). The other alternative human GFRα4 transcripts are:

(ii) GFRA4b (900 bp), in which the small intron (79 bp) separating exons 2 and 3 (Figure 9A) is included in the transcript, and the 3'splice site of exon 4 is located 11 bp upstream of the respective splice site used in the GFRA4a transcript. The small intron between exons 2 and 3 is also inefficiently spliced in majority of mouse tissues. Inclusion of this intron in mouse Gfra4 transcripts with exon 1a or exon 1b leads to putative soluble protein isoform. In contrast, translation of the transcript GFRA4b in human would lead to a GPI-linked protein of 299 amino acids. In this protein isoform, the N-and C-terminal ends are identical to the respective regions of GFRA4a, but the middle region consists of a stretch of 66 amino acids translated in different frame from intron 2 and exon 3. This sequence is not homologous to any protein in public databases.

(iii) GFRA4c (867 bp), in which the introns between exons 2 and 3, and between exons 3 and 4 are included in the transcript. These introns would lead to a frameshift with a stop codon located inside exon 5 and production of a putative soluble isoform of 236 amino acids. It is interesting to note that the sequences of the 3'splice sites of both mouse and human exon 3 and exon 4 contain a short polypyrimidine-tract interrupted with purines, which is characteristic to alternatively spliced exons (Lou, et al., 1994).

The present invention is also related to expression of GFRA4, PSPN and RET in different human tissues. Expression of GFRA4, PSPN and RET was analyzed by RT-PCR in 25 different adult and two fetal tissues (Figure 10). Expression of GFRA4 was only

detected in the adult thyroid gland (Figure 10) using primers P2 and P3 (see Figure 9). Similar results were obtained using different combinations of primers (data not shown). The smallest band of 699 bp corresponds to the GFRA4a cDNA where all introns are spliced out, whereas the 789 bp band corresponds to the intron 2-containing GFRA4b transcript. The 863 bp PCR fragment corresponds to the cDNA containing both introns 2 and 3 encoding the putative soluble isoform (GFRA4c, Figure 9B). The unspliced transcript (approx. 589 bp) of PSPN was expressed in all human tissues examined using primers detecting the full-length PSPN transcript (Figure 10, row 2). No transcripts were observed when RNA was used as a template for PCR, which shows that the cDNA samples did not contain chromosomal DNA. Low levels of spliced PSPN transcripts (476 bp) encoding the functional protein were present in human adrenal gland, cerebellum, spinal cord and testis, and were not detected in the thyroid gland. RET mRNA was expressed at variable levels in most tissues examined (Figure 10, row3).

The present invention is related to activation of human GFRα4/Ret receptor complex, which is unique for PSPN. To study the binding of human PSPN to human GFRα4, we used GFRA4a-transfected mouse neuroblastoma Neuro-2a cells (hGFRα4/Neuro-2a), which endogenously express Ret. These cells strongly bound [^{125}I]PSPN, while mock-transfected Neuro-2a cells did not (Figure 11C). Low concentrations (Approx. 1 nM) of unlabeled PSPN effectively displaced [^{125}I]PSPN from the GFRA4-expressing cells, whereas GDNF (up to 300 nM), NRTN (up to 200 nM) and ARTN (up to 200 mM) were ineffective (Figure 11A). Thus, in the presence of Ret human GFRα4 binds specifically PSPN, but not the other GDNF family ligands. PSPN binds to human GFRα4 with IC50 of 1 nM, and a dissociation constant (KD) of approximately 100 pM (Figure 11A).

Next we studied the binding of PSPN to GFRA4a-transfected CHO cells, which do not express Ret. Treatment of the cells with [^{125}I]PSPN, followed by chemical cross-linking and analysis by

SDS-PAGE, resulted in a major band of approx. 46 kDa and minor bands of approx. 62 and 92 kDa (Figure 11B). The approx. 46 kDa band corresponds a PSPN monomer (approx. 14 kDa) cross-linked to monomeric GFRα4 (30 kDa). The approximately 62 and 92 kDa bands most probably correspond to PSPN dimer cross-linked to GFRα4 monomer and dimer, respectively. Additional 16 and 32 kDa bands represent PSPN monomer and dimer, respectively (Figure 11B, lane 6). No specific bands were detected from mock-transfected cells (Figure 11B, lanes 1 and 2). The amount of cell-bound complexes was greatly reduced by adding unlabeled PSPN or by removal of the GPI-anchored proteins by phosphoinositide-specific phospholipase C (PI-PLC) after cross-linking (Figure 11B, lane 4 and 5). Combination of these two treatments further reduced the yield of cross-linked products to undetectable levels (Figure 11B, lane 3). Thus, the human GFRα4 protein, encoded by the GFRA4a transcript indeed contains a GPI anchor. Cross-linked complexes of the same sizes were also identified from hGFRα4/Neuro-2a cells (Figure 11C). In addition, a minor band of about 200 kDa was observed (Figure 11C) that could be a complex of PSPN/GFRα4 with Ret.

To study the interaction of PSPN with Ret, proteins cross-linked to [^{125}I]PSPN in hGFRα4/Neuro-2a cells were precipitated with Ret antibodies (Figure 11D, lanes 2 and 4). The major cross-linked complexes of approximately 196 and 232 kDa, as well as minor complexes of about 62 and 92 kDa (same size as bands in Figure 11C), and also of approx. 400 kDa were obtained under reducing conditions (Figure 11D, lane 2). Under non-reducing conditions, the approx. 400 kDa band was greatly intensified, which indicates the presence of S-S-bound complexes. No cross-linked cell-bound products were detected using parental Neuro-2a cells (Figure 11D, lanes 1 and 3). The bands of about 196 and 232 kDa correspond to the complexes of PSPN/Ret (186 kDa) and PSPN/GFRα4/Ret (216 kDa), respectively. The components of the complex of about 400 kDa could be a dimer of the 186 kDa PSPN/Ret complex.

To determine whether PSPN binding to human GFRα4 mediates Ret autophosphorylation, hGFRα4/Neuro-2a cells treated with PSPN were used. Gfra1 expressing Neuro-2a cells treated with GDNF served as a positive control. PSPN induced Ret tyrosine autophosphorylation in hGFRα4/Neuro-2a cells, which showed a phosphorylated band of 170 kDa, corresponding to the active form of Ret (Figure 11E). No phosphorylation of Ret was observed in vector-transfected Neuro-2a cells (Figure 11E, mock). Stimulation of Ret phosphorylation was dose-dependent starting at 0.1 ng/ml of PSPN (Figure 11F).

The present invention is related to PSPN which specifically promotes survival of neurons ectopically expressing GFRα4 and RET. In order to study whether binding of PSPN to mammalian GFRα4/Ret complex triggers functional cellular programs, as described for chick GFRα4 (Enokido, et al., 1998). The present inventors expressed the GPI-linked mouse Gfra4 or human GFRA4a together with human RET in neonatal mouse SCG neurons and maintained the neurons further with PSPN for three days. SCG neurons are trophically dependent on NGF, do not express full-length Gfra4 (not shown) and cannot be trophically supported by PSPN (Enokido, et al., 1998; Lindahl, et al., 1999; Milbrandt, et al., 1998). Significant portion of neurons expressing mouse GFRα4 and RET was maintained by PSPN (60% versus 16% without PSPN, $p<0.001$), whereas omission of GFRα4 abolished this trophic effect (Figure 12). Similar results were obtained with human GFRA4a (not shown). Also GDNF and NRTN maintained part of neurons expressing RET and Gfra4, but these effects were not reduced by omission of Gfra4 (Figure 12). Moreover, both factors maintained also uninjected neurons similarly to receptor-injected neurons (Figure 12). ARTN promoted survival of neither GFRα4/RET injected nor uninjected mouse SCG neurons (Figure 12). Thus, GFRα4/RET complex requires PSPN, but not other GDNF family members, to activate a survival-promoting program in SCG neurons.

The present invention is related to selective expression of GFRA4 and RET mRNAs solely in medullary thyroid tumours. Co-localization of Gfra4 and Ret in developing and mature mouse thyroid C-cells and their co-expression in adult human thyroid tissue (Figure 10), prompted us to study the expression of GFRA4, RET and PSPN mRNAs in human primary thyroid tumors. RT-PCR analysis of GFRA4 expression, using primers generating full-length transcript, showed that similar levels of GFRA4 mRNAs encoding the two GPI-linked isoforms and lower levels of transcripts encoding the soluble isoform, are expressed in all eight MTC samples analyzed (Figure 13, row 1). GFRA4 mRNA was not detected in any of the follicular thyroid adenomas (FTA), follicular thyroid carcinomas (FTC) or papillary thyroid carcinomas (PTC) analyzed. Cellular localization of GFRA4 mRNA was studied by *in situ* hybridization in the same thyroid tumor samples (Figures 14A and 14B). GFRA4 was highly expressed by virtually all the malignant C-cells in the MTC samples analyzed, but not in the accompanying connective tissue and blood vessels. No expression of GFRA4 was detected in the adjacent apparently normal follicle cells found in some samples. Other thyroid tumors, including FTA, FTC and PTC did not express GFRA4. None of the thyroid tumors expressed detectable levels of spliced PSPN mRNA, whereas the unspliced form of PSPN transcript was present in all tumor samples analyzed (Figure 13, row 2). High levels of RET expression was seen in all MTC tumors (Figure 13, row 3 and Figure 14).

Tumor samples were further analyzed for GFRA1, GFRA2 and GFRA3 mRNA expression by RT-PCR and *in situ* hybridization (Figure 14A and Table 2). In some tumor samples, low levels of GFRA1, GFRA2 and GFRA3 mRNAs were present in subsets of the tumor cells. To study if GFRA4 gene is mutated in the MTC tumors, full-length GFRA4 cDNA clones from eight MTC samples, the TT cell-line, and normal thyroid were sequenced. Except for a few polymorphisms in the coding region, which do not change the amino acid composition of GFRα4, no mutations were found in

the MTC samples or in the TT cell-line. Thus, the coding region of GFRA4 gene is not mutated in MTC tumors.

The present invention is related to a PSPN-GFRα4-Ret complex inducing only the PI3-K/Akt pathway. To test the possible difference between GDNF-GFRα1-Ret and PSPN-GFRα4-Ret signalling mouse neuroblastoma Neuro-2a cell lines stable transfected with either GFRα1 or hGFRα4. Neuro-2a cells express endogenous Ret. Stimulation of Neuro-2a/GFRα1 cells with GDNF lead to the activation of MAP (Figure 16A) as well as Akt kinases (Figure 15A). Stimulation of Neuro-2a/hGFRα4 cells with PSPN also lead to the Akt activation (Figure 15B), however, the level of MAPK phosphorylation remained unchanged (Figure 16B). This difference between GDNF/GFRα1 and PSPN/hGFRα4 evoked responses appeared also at the physiological level. GDNF and PSPN, acting via their respective co-receptors, both were able to stimulate Neuro-2a cell survival in serum-free conditions (Figure 17A). In another assay, GDNF but not PSPN was able to induce the neurite outgrowth from the Neuro-2a cells transfected with either GFRα1 or hGFRα4, respectively (Figure 17B). These results suggest that while GDNF-GFRα1-Ret system activates both Ras/MAPK and PI3-K/Akt cascades, the PSPN-GFRα4-Ret complex can induce only the PI3-K/Akt pathway. PSPN-GFRα4-Ret signalling may be unable to activate the MAPK activity or it may be suppressed during this signalling. Consequently, GFRα1- and GFRα4-specific pathways lead to either mainly differentiation or survival.

In the present invention it was also shown that unlabelled PSPN displaces [^{125}I] PSPN from GFRA4-transfected Neuro-2a cells, which express endogenous Ret. PSPN can be specifically cross-linked to mammalian GFRα4 and Ret, and is able to promote autophosporylation of Ret in GFRA4-transfected cells. Furthermore, it is shown that PSPN, but not other GDNF family ligands, promotes the survival of cultured sympathetic neurons microinjected with GFRA4 and RET.

In the present invention it has further been shown that GFRα4 is the functional receptor for PSPN. The binding and cross-linking studies demonstrated that association of PSPN with the Ret receptor protein tyrosine kinase is mediated by GFRα4. Ligand displacement binding showed that in the presence of Ret, only PSPN but not GDNF, NRTN or ARTN was effective in displacing [^{125}I]PSPN from GFRA4-expressing Neuro-2a cells. The dissociation constant (KD) of about 100 pM found here is ten times lower than the KD of about 1 nM reported for mouse PSPN binding to chicken GFRα4 (Enokido, et al., 1998), and 60 times lower than the about 6 nM reported for rat PSPN binding to immobilized rat GFRα4 fusion protein reported by Masure, et al., 2000, but similar to those reported for GFRα1, GFRα2 and GFRα3 and their cognate ligands (Klein, et al., 1997; Trupp, et al., 1998; Baloh, et al., 2000). The lower binding of mammalian PSPN to chicken GFRα4 probably reflects the species difference in GFRα4 structure. PSPN has not yet been characterized from chicken and, if it exists, might differ significantly from the mammalian PSPN.

The cross-linking studies show that PSPN is able to bind GFRα4 also in the absence of Ret. This is consistent with the model that a GDNF family ligand first binds to the corresponding GFRα receptor and subsequently the ligand-GFRα complex binds to Ret (Jing, et al., 1996) However, our results also agree with the alternative model in which the ligand binds a preformed GFRα/Ret complex (Sanicola, et al., 1997). Although PSPN binding to chicken GFRα4 has been shown earlier (Enokido, et al., 1998).

In the present invention it is demonstrated for the first time that the association of PSPN with GFRα4 results in activation of the Ret tyrosine kinase. PSPN treatment of cells expressing GFRα4 rapidly induced Ret autophosphorylation in a dose-dependent manner. PSPN is unable to stimulate Ret autophosphorylation in cells that do not express GFRα4. Thus,

the GFRα4a isoform characterized in the present invention is a functional receptor for PSPN in triggering Ret activation. In contrast to this, the rat GFRα4 isoform with a poor signal sequence described by Masure et al., 2000 bound PSPN only as soluble fusion protein and did not lead to Ret activation.

In the present invention it has also been demonstrated that PSPN but not other GDNF family ligands, can promote the survival of cultured SCG neurons ectopically expressing Gfra4 and RET. In our assay, neurons co-expressing RET plus GFRα4 showed an elevated response to GDNF and NRTN but not to ARTN, however, also the uninjected neurons responded to GDNF and NRTN. Therefore, it seems that GDNF and NRTN maintain these neurons via activation of endogenous binding sites and not via GFRα4. Taken together, the binding of PSPN to GFRα4/Ret complex triggers functional cellular responses.

In the present invention the putative role for GFRα4 in medullary thyroid carcinoma is demonstrated. As the RET proto-oncogene plays an important role in the oncogenesis of MTC, a logical step was to examine GFRα4 expression in these tumors. GFRA4 transcripts encoding the two GPI-anchored isoforms were expressed at high level in all MTC samples, whereas no GFRA4 expression was detected in any other type of thyroid tumor analyzed. In addition, moderate levels of GFRA4c encoding the putative soluble form of GFRα4 were found in all MTC samples. Taken together, the data provided by the present inventors suggest that MTC-specific expression of GFRA4 could have diagnostic value.

Strong RET expression was also localized to the malignant C-cells consistent with previous reports (Nakamura, et al., 1994; Santoro, et al., 1990). Low levels of GFRA1, GFRA2 and GFRA3 transcripts were present in subsets of tumor cells, indicating that their expression is not specific to particular thyroid tumor cell type. This result is in line with a recent report showing GFRA1 and GFRA2 expression in some MTC tumor

cells (Frisk, et al., 2000). However, our results showing low GFRA1 and GFRA2 expression also in normal thyroid, in other thyroid tumor types, and not in all MTC samples, does not support the idea that GFRα1 or GFRα2 play a role in the primary pathogenesis of MTC. Phenotypic variability within the same MEN2 family or between different families carrying the same mutation in RET suggest that further genetic events or modifier genes are required to induce the tumor phenotype in MTC. The results of the present invention indicate that co-expression of GFRα4 with mutated RET may be necessary for the initial hyperplasia of C-cells occurring in MTC. It should be possible to test this hypothesis by examining whether the development of C-cell hyperplasia and subsequently MTC, pheochromocytoma and ganglioneuromas are suppressed in Gfra4-deficient mice crossed with MEN2 transgenic mice (Smith-Hicks, et al., 2000; Acton, et al., 2000; Laurikainen, et al., 2000).

Further studies with metastases derived from MTC tumors and other endocrine tumors such as GFRα4 expressing pheocromocytomas provide more evidence as regards these questions. Further *in vitro* studies on cell lines and primary thyroid C-cells should also be informative to reveal the roles for PSPN-signalling via different GFRα4 isoforms through normal and mutant RET.

Similar results were obtained from all thyroid tumours analyzed: GFRA4 was highly expressed in all tumour cells of 6 medullary carcinoma samples but not detectable in cells from other thyroid tumours including tissue samples from 4 follicular adenomas, 4 follicular carcinomas and 5 papillary carcinomas. samples. The six medullary carcinomas analyzed included both sporadic MTC, as well as tumors from patients with inherited MEN2 syndrome (carrying different mutations in RET).

As a conclusion the present invention is related to mammalian,

but especially human GFRα4 and GFRα-like molecules and/or compounds interacting with said compounds, where GFRα4 is the ligand-binding subunit required together with RET for PSPN signalling.

The invention provides isolated and purified nucleic acid sequences (e.g. cDNAs) comprising a nucleotide sequence encoding a GFRα4 protein or a fragment thereof. Also described are fragments of said nucleotide sequences, which are suitable as primers or hybridization probes for the detection of GFRα4-encoding nucleic acid sequences (e.g. mRNA). The isolated nucleic acid sequences encode the murine amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) and the human amino acid sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:), particularly the murine nucleotide sequences (SEQ ID NO:7), (SEQ ID NO:8:) or (SEQ ID NO:9:) and the human nucleotide sequences (SEQ ID NO:10:), (SEQ ID NO:11:),(SEQ ID NO:12:), (SEQ ID NO:13:).

The isolated nucleic acid sequences of the present invention hybridize under stringent conditions to sequences which are at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 98% or more homologous to the nucleic acid sequences defined in the claims.

The GFRα4 protein or polypeptide fragments or a biologically active portion thereof can be operatively linked to a non-GFRα4 polypeptide to form a fusion protein.

The isolated and purified nucleic acid sequences encode proteins or fragments thereof including amino acid sequences which are sufficiently homologous to a protein comprising the murine amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) as well as the human amino acid sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) as long as the protein or the fragment thereof maintains the specific GFRα4 activities described above. Preferably, the GFRα4-protein or

fragments should maintain the ability to bind to a neurotrophic factor and modulate a cellular response in the way defined in the claims and as described above. The protein encoded by the nucleic acid sequences should be at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 98% or more homologous to the murine amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) or the human amino acid sequences (SEQ ID N0:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) or the fragments (SEQ ID NO:14:), (SEQ ID NO:15:), (SEQ ID NO:16:) or (SEQ ID NO:17:).

The protein should preferably be a full length protein which is substantially homologous to the entire murine amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) or the entire human amino acid sequences (SEQ ID N0:4:), (SEQ ID NO:5:), (SEQ ID NO:6:) or the fragments (SEQ ID NO:14:), (SEQ ID NO:15:), (SEQ ID NO:16:) or (SEQ ID NO:17:) and includes all naturally occurring full length proteins and allelic variants and splice variants of human or murine GFRα4.

The isolated GFRα4s should preferably comprise amino acid sequences which are at least about 70-80% or more homologous to the murine and human amino acid sequences defined above and should be characterized by at least one but preferably several of the following activities as well as those described in the discussion of the experiments disclosed above:

1) it can interact with (e.g. bind to) a neurotrophic factor.
2) it can interact (e.g. bind to) a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET;
3) it can modulate the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET; and
4) it can bind a neurotrophic factor and modulate a response in endocrine cells, e.g., thyroid C-cells.

The isolated nucleic acid sequence should comprise at least 15-20 nucleotides capable of hybridizing under stringent

conditions to a nucleic acid sequence comprising the one of the murine nucleotide sequences (SEQ ID NO:7:), (SEQ ID NO:8:) or (SEQ ID NO:9:) or the human nucleotide sequences (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:) or parts thereof.

Preferably, the isolated and/or purified nucleic acid sequence corresponds to the naturally occurring nucleic acid sequence. More preferably, the isolated or purified nucleic acid sequences should encode naturally-occurring alleles and splice variants of human GFRα4. Moreover, the GFRα4-encoding murine cDNA sequences (SEQ ID NO:7:), (SEQ ID NO:8:) or (SEQ ID NO:9:) or the human cDNA sequences (SEQ ID NO:10:), (SEQ ID NO:11:) or (SEQ ID NO:12:) or (SEQ ID NO:13:) or cDNA fragments (SEQ ID NO:18:), (SEQ ID NO:19:), (SEQ ID NO:20:), (SEQ ID NO:21:), (SEQ ID NO:22:), (SEQ ID NO:23:), (SEQ ID NO:24:), (SEQ ID NO:25:), (SEQ ID NO:26:), (SEQ ID NO:27:), (SEQ ID NO:28:), (SEQ ID NO:29:), (SEQ ID NO:30:), (SEQ ID NO:31:), (SEQ ID NO:33:), (SEQ ID NO:35:), (SEQ ID NO:37:), (SEQ ID NO:39:), (SEQ ID NO:40:), (SEQ ID NO:41:), (SEQ ID NO:42:), (SEQ ID NO:43:), (SEQ ID NO:44:), (SEQ ID NO:45:), (SEQ ID NO:46:), (SEQ ID NO:47:), (SEQ ID NO:48:), (SEQ ID NO:48:) or (SEQ ID NO:50:), as well as strands which are complementary to the coding strand of the GFRα4 cDNA sequences.

The invention is also related to recombinant transformation or expression vectors or constructs as well as procaryotic or eucaryotic, including mammal host cells comprising one or more of the nucleic acid sequences defined in the claims. Such host cells may be used to produce GFRα4 proteins by culturing the host cell in a suitable medium. If desired, the GFRα4-protein can then be isolated from the host cell.

The invention is also related to transgenic non-human animals in which a *GFRα4* gene has been introduced or altered. In such cases the genome of the non-human animal, e.g. a mouse, rat,

guinea pig, etc., can be altered by introduction of a nucleic acid sequences of the invention encoding GFRα4 as a transgene. An endogenous *GFRα4* gene within the genome of the non-human animal can be altered or made functionally incompetent by homologous recombination or by using the knock-out technique.

The invention is further related to isolated GFRα4 or GFRα4-like molecules or compounds interacting with said molecules, e.g. biologically active fragments thereof. The isolated GFRα4s or fragments can bind to a specific neurotrophic factor and stimulate a response in a neurotrophic factor responsive cell.

The invention also provides preparations or compositions of GFRα4s which comprise amino acid sequences of the GFRα4s defined above in combination with carriers or diluents which can be pharmaceutically acceptable or not depending upon the application.

The GFRα4s of the present invention can be used to prepare anti-GFRα4 antibodies. Accordingly, the invention also provides antigenic peptides of GFRα4. These should preferably comprise at least 8 amino acid residues of the murine or human amino acid sequences defined above and in the claims. These should encompass at least one epitope of GFRα4 against which an antibody may be raised.

The peptide may form a specific immune complex with GFRα4. Preferably, the antigenic peptide comprises at least 10, more preferably 15, at least 20 or more amino acid residues providing a sufficiently high antigenicity index. Thus the present invention provides antibodies which specially bind GFRα4 or GFRα4-like molecules or compounds interacting with said molecules. The antibody is preferably monoclonal, but naturally polyclonal antibodies may be used. The antibody may be coupled to a detectable or recordable substance and it may be incorporated into a pharmaceutical composition comprising

the antibody and a pharmaceutically acceptable carrier.

Furthermore, the invention pertains to methods for modulating the GFRα4 mediated cell activity or signalling including cell proliferation and/or differentiation. Such methods include contacting the cell with a compound or agent capable of modulating the activity of a GFRα4 protein or the expression of *GFRα4* nucleic acid sequence in such a way that a cell associated activity is measurably altered in relation to the cell associated activity in the absence of the agent. The cell, e.g. a neuron or endocrine cell should be capable or responding to a neurotrophic factor through a specific signalling pathway involving the GFRα-4 protein, as described in the examples.

The agents, which modulate GFRα4 protein can be agonists, which stimulate GFRα4 activity or the expression of the *GFRα4* nucleic acid sequences or antagonists, which inhibit GFRα4 protein activity or expression of *GFRα4* nucleic acid sequence.

Examples of agents which stimulate GFRα4 protein activity or *GFRα4* nucleic acid expression include small molecules and nucleic acid sequences encoding GFRα4 which can be or have been introduced into the cell. Examples of agents which inhibit GFRα4 activity or expression include small molecules, antisense *GFRα4* nucleic acid sequences, antibodies specifically binding to GFRα4. The agent is administered to a cell, such as a neuron or an endocrine cell present in the subject.

The present invention also pertains to methods for making diagnoses and/or treating subjects having disorders mediated by abnormal GFRα4 activity and/or expression. For example, the invention pertains to methods for treating a subject having a disorder characterized by aberrant GFRα4 protein activity or nucleic acid expression. Examples of such disorders are

neuronal disorders, endocrine system disorders, e.g., multiple endocrine neoplasia type 2 (MEN2) syndrome, disorders associated with abnormal or aberrant cell development, differentiation and function. For example, axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures may aggravate the disease process. Augmenting GFRα4 type of signaling in such conditions could be useful by preventing neuronal death without producing aberrant sprouting.

The methods of treatment include administering to the subject GFRα4 or GFRα4-like molecules or compounds interacting (modulators or mimetics) with said molecules in such amounts that treatment of the subject occurs.

The invention also pertains to methods for detecting genetic mutations in a *GFRα4* gene, thereby determining if a subject with a mutated gene is at risk of having or is predisposed to have a disorder characterized by aberrant or abnormal *GFRα4* nucleic acid expression or GFRα4 activity, such as an endocrine system disorder, rare types of MEN2-like syndromes without *Ret* mutations or MEN2-like syndromes with additional phenotype, e.g., pituitary tumours. Activity of GFRα4 signalling pathway in endocrine systems can lead to disorders including tumours associated with cellular development of cells of these organs as shown in the present RET invention. For example, axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures may aggravate the disease process. Augmenting GFRα4 type of signaling in such conditions could be useful by preventing neuronal death without producing aberrant sprouting.

The methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic mutation characterized by an alteration affecting the integrity of a gene encoding a GFRα4 protein, or the misexpression of the *GFRα4* gene.

The invention further pertains to methods for detecting the presence of GFRα4 or fragment thereof, in a biological sample. The methods involve contacting a biological sample, e.g. any (metastatic) tumour sample, with a compound or an agent capable of detecting GFRα4 protein or GFRα4 encoding mRNA in such away that the presence or abscence of GFRα4 can be detected in the biological sample. The compound or agent can be, for example, a nucleic acid probe which is or can be labelled and is capable of hybridizing to GFRα4 encoding mRNA or an antibody which is labelled or can be labelled with a suitable detectable marker and is capable of binding to a GFRα4 protein. The invention further provides methods for diagnosing the subject with, for example, an endocrine system disorder or neuron outgrowth disorder based on detection of GFRα4 protein or its mRNA as well as the GFRα4 mediated signalling.

The method involves for example the contacting of a cell, tissue, or fluid sample (e.g., a tumour sample) from the subject with an agent capable of detecting GFRα4 protein or its mRNA and thereafter determining the amount of GFRα4 protein or mRNA expressed in the sample to a control sample and forming a diagnosis based on the amount of GFRα4 protein or mRNA expressed in the sample or its signalling as compared to the control sample. Kits for detecting GFRα4, or fragments thereof by immunoassays or hybridization or microarray techniques from a biological sample are thus also within the scope of the invention.

The present invention also relates to methods, especially, screening assays for identifying compounds or identifying disorders characterized by aberrant *GFRα4* nucleic acid expression or protein activity. Activity of GFRα4 signalling pathway in endocrine systems can lead to disorders including tumours associated with cellular development of cells of these organs as shown in the present RET invention. For example,

axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures may aggravate the disease process. Augmenting GFRα4 type of signaling in such conditions could be useful by preventing neuronal death without producing aberrant sprouting.

These methods typically include assaying the ability of a compound or agent to modulate the expression of the *GFRα4* gene or the activity of the GFRα4 mediated signalling and thereby identifying compounds which are useful for treating a disorder characterized by aberrant *GFRα4* nucleic acid expression or protein activity. The method involves contacting a biological sample obtained from a subject having the disorder with the compound or agent, determining the amount of GFRα4 protein expressed and/or measuring the activity of the GFRα4 protein in the biological sample, comparing the amount of GFR4 protein expressed in the biological sample and/or the measurable GFR4 biological activity in the cell to that of a control sample. An alteration in the GFRα4 expression or GFRα4 mediated signalling in a cell exposed to a compound or agent in comparison to the control is indicative of a modulation of GFRα4 expression and/or GFRα4 activity.

The invention also pertains to methods for identifying compounds or agents which interacts with (e.g., binds to) or mimicks a compound binding to or vice versa a GFRα4 protein. These methods can include the steps of contacting the GFRα4 protein or fragments thereof or alternatively a cell expressing GFRα4 with the compound or agent under conditions which allow binding of the compound to the GFRα4 protein to form a complex. Thereafter, the formation of the complex comprising the GFRα4 protein and the compound having the ability of specifically binding to the GFRα4 protein is measured. The formation of the complex is an indication of the presence of a potentially useful interacting compound. Applicable methods are described in the following patents and patent applications EP 639 584, WO 9638553, US 5,571,506,

WO 200018790, WO 9950439, WO 9945930, US 0,630,619 and WO 9906599.

The invention further pertains to methods for identifying a compound or agent which can modulate, stimulate or inhibit the interaction of the GFRα4 protein with a target molecule, such as persephin or other neurotrophic factors and the tyrosine kinase receptor RET or other components of signalling pathways and their complexes. In these methods, the GFRα4 protein is contacted, in the presence of the compound or agent, with the target molecule under conditions which allow binding of the target molecule to the GFRα4 protein to form a complex. An alteration, e.g., an increase or decrease, in complex formation between the GFRα4 protein and the target molecule as compared to the amount of the complex formed in the absence of the compound or agent is indicative of the ability of the compound or agent to modulate the interaction of the GFRα4 protein with a target molecule.

The invention is also related to substances capable of specifically recognizing the GFRα4 polypeptides defined in above and in the claims. Such substances are for example antibodies, including polyclonal, monoclonal antibodies produced by recombinant DNA techniques or hybridoma techniques as well as ligands capable of binding specifically to the mammalian GFRα4-receptor. Fragments of antibodies and receptors are also useful as specifically binding substances.

The present invention also enables the production of transgenic non-human animal lacking a functional GFRα4 receptor and which is useful as a test animal obtainable from a genetically modified compatible cell-line (clone) containing a genomic gene coding for a functional GFRα4 receptor, which genomic gene may be functionally inactivated or conditionally inactivatable. Said transgenic non-human animal is useful as a test animal or model for studying GFRα4-mediated signalling in

neuronal cells, endocrine cells, especially in thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, nervous system and testis, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia and for diagnosing and/or treating osteoporosis, endocrine tumours, cancer in parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia. Activity of GFRα4 signalling pathway in endocrine systems can lead to disorders including tumours associated with cellular development of cells of these organs as shown in the present RET invention. For example, axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures may aggravate the disease process. Augmenting GFRα4 type of signalling in such conditions could be useful by preventing neuronal death without producing aberrant sprouting.

The present invention also provides a method for obtaining a cell-line (clone) useful in the production of the transgenic non-human animal characterized by having the genomic gene encoding a functional GFRα4 receptor inactivated by introducing into stem cells a nucleic acid sequence, which is capable of integrating to said genomic *Gfra4* gene and by said integration functionally inactivating said genomic gene or making it conditionally inactivatable.

The cell-line (clone) lacking a functional GFRα4 receptor is obtainable by established gene targeting methods, by:
(a) providing a nucleic acid sequence defined in the claims, which sequence is capable of being integrated into the genomic gene coding for a functional GFRα4 receptor;
(b) preparing a targeting construct by combining the nucleic acid sequence according to step (a), with a selectable nucleic acid sequence acting as a marker which is capable of functionally inactivating and by said integration functionally

inactivating the genomic gene or making it conditionally inactivatable;
(c) introducing the targeting construct or vector of step (b) into stem cells or cell lines (clones) of the selected animal;
(d) selecting a cell line (clone) containing the nucleic acid sequence or part thereof according to step (a) which has been integrated into the genomic gene of said stem cell or cell line (clone) using a probe, which is capable of recognizing the selectable nucleic acid sequence acting as a marker.

A cell line (clone) obtained by the method described above is lacking a functional GFRα4 receptor and produces consequently altered levels of GFRα4. The cell lines (clones) of the present invention are useful not only for producing a transgenic non-human animal but also for studying the effect of different compounds, e.g. obtained by combinatorial chemistry, on GFRα4-mediated signalling in neurons, endocrine cells, especially in thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, nervous system and testis, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia and for diagnosing and/or treating osteoporosis, endocrine tumours, cancer in parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia.

The invention is described in more detail in the following examples which are intended to illustrate the invention not to limit its scope.

Example 1
Sequence analysis

Several putative exon sequences homologous to known Gfra genes

were identified by BLAST analysis of a 47887 bp sequence containing the putative mouse *Gfra4* locus (Gunn et al., 1999) (Genebank accession number AF155960). Database search using these sequences as query detected three mouse expressed sequence tags (ESTs) matching the 3' end of the putative Gfra4 gene (AA823200 or AI1553571, a spliced EST from mammary gland; AU035938, an unspliced one from the brain; and AI845209 a short one from the spinal cord). Nucleotide and amino acid sequence comparisons (BestFit), alignment (PileUp), and phylogenetic tree (GrowTree), as well as signal sequence and transmembrane hydrophobicity plots (PeptideStructure) were analyzed using the SeqWeb program package (Genetics Computer Group, Inc.).

Example 2
RACE cloning

cDNA clones representing the 5'-region of *Gfra4* transcript were isolated from adult mouse brain, testis and thyroid gland, as well as from E17 embryo, using the 5'-RACE System (Life Technologies) and the GC-RICH PCR kit (Roche) according to the manufacturer's instructions. Total RNA was extracted with Trizol reagent (Life Technologies). The anti-sense *Gfra4* specific primers used in 5' RACE were: 5'-TTC AGC TCA GTG AGC AGT CAT CG-3' (SEQ ID NO:18:) and second nested primer 5'-CAG GTT GTC CAG GTA GTT GG-3' (SEQ ID NO:19:) or 5'-CAT CGT GCC ACG TAC TCA GA-3'(SEQ ID NO:20:). The 3' region of the *Gfra4* was cloned with 3'RACE method using Clontech Marathon Ready cDNA from E15 mouse embryos according to the manufacturer's instructions. The primers used in 3'-RACE were: 5'-TAC AAG CCT TTG ACA GCT TGC AGC-3' (SEQ ID NO:21:) and a nested one 5'-AGA GCT GGA GGC AGA AAC AGT CC-3' (SEQ ID NO:22:).

Human GFRA4 cDNAs were identified from a human thyroid cDNA (Clontech Marathon Ready cDNA) by 3'and 5'RACE method using the GC-rich PCR kit (Roche) according to the manufacturer's instructions. Reverse transcription reactions were performed

using Superscript II (Life Technologies). PCR systems were GC-RICH PCR kit (Roche) for GFRA4 and PSPN, Expand Long Template PCR system (Roche) for GFRA1, GFRA2, GFRA3 and RET and Dynazyme II (Finnzymes) for PPIA (alias cyclophilin). PCR was run 40 cycles for GFRA4, 35 cycles for PSPN, GFRA1 and GFRA2, and 30 cycles for GFRA3, RET and PPIA using annealing temperatures of 55-60(C. Full-length human GFRA4 cDNA was obtained using Expand High Fidelity system PCR enzyme (Roche) together with GC- RICH PCR system buffers (Roche). PCR products were cloned into mammalian expression vector pCR3.1 (Invitrogen).

Example 3
Northern Blot and Reverse Transcription (RT)-PCR

Norther blot analysis of *Gfra4* expression was carried out from total RNA essentially as described (Rossi et al., 1999). Analysis of the splicing pattern of *Gfra4* was performed by RT-PCR using Superscript II (Life Technologies) for RT and the GC-RICH PCR kit (Roche) for PCR. The following primers were used (see Figure 2C): P1, 5'-CCA CCA TGG CCC ACT GCA TGG AGTC-3' (SEQ ID NO:23:); P2, 5'-CCA CCA TGT TGA GAA GAG CA-3' (SEQ ID NO:24:); P3, 5'-GTG TAC AGC AGA CGA GCG GT-'3' (SEQ ID NO:25:); P5, 5'-ATA CAA GCC TTT GAC AGC TTG C-3' (SEQ ID NO:26:); and reverse primers P4, 5'-GTT CCT TGT AAA GAG CTT GCG-3' (SEQ ID NO:27:); and P6, 5'-TGG ACA AGA TGC CTA CTG ACG-3' (SEQ ID NO:28:). The amounts of total RNA was run 35-40 cycles using annealing temperatures of 55-58°C. All the PCR products of different length were sequenced to verify their identity.

Example 4
RNAse Protection Analysis

RNAse protection assays were performed as described (Timmusk et al., 1993) using the RPAII kit of Ambion according to manufacturer's instructions. The template for *Gfra4* specific

cRNA probe was a 550 bp PCR fragment generated using primers P3 and P4. It yielded a fully protected fragment corresponding to *Gfra4* mRNA that have spliced both introns between exons II and IV, as well as 235 bp and 315 bp fragment corresponding to *Gfra4* mRNA that is unspliced between exons II and III.

Example 5
***In Situ* Hybridization**

In situ hybridizations for *Ret*, Gfra1 and *Gfra2* was performed as described Rossi et al., 1999). A mouse full-length *Gfra3* cDNA (AF051766) and a bp cDNA fragment of the 3'-end of the mouse *Gfra4* was used as template for sense and anti-sense RNA probes. Control sections hybridized with sense probe did not show labeling above background (not shown).

In situ hybridizations for RET, GFRA1, GFRA2, GFRA3 and GFRA4 were performed principally in the same way as described above on cryosections of the same thyroid tumor samples analyzed by RT-PCR. A 206 bp cDNA fragment of the 5'-end of human GFRA4 was used as template for sense and antisense RNA probes. PCR fragments of GFRA1, GFRA2, GFRA3 and RET generated with the same primers as in RT-PCR were cloned and sequenced and used as templates for antisense and sense RNA probes. Control sections hybridized with sense probe did not show labeling above background (not shown). Dark-field and corresponding bright-field images of Nissl counter-stained sections were digitized and processed using Adobe PhotoShop software.

Example 6
Immunohistochemistry

Ret-deficient (-/-) and control littermate mice were genotyped as described (Durbec et al., 1996). Anesthetized day E18 embryos were perfused transcardially with 4% paraformaldehyde in PBS, postfixed for 2 hours and cryoprotected overnight in 25% sucrose. Horizontal cryosections of the thyroid gland,

serially sectioned at 15μm, were stained and analyzed by standard immunofluorescence techniques. DAPI (Sigma) was included in embedding medium to stain the cell nuclei. The primary goat polyclonal antibody against calcitonin (sc-7784) was from Santa Cruz Biotechnology. All immunopositive profiles with a clear nucleus were counted in every section through the thyroid glands. The mean nuclear diameter of the C-cells was similar (<1% difference) between the *Ret* -/- and control groups (not shown). Raw profile numbers per animal without correction are given as mean ± S.D.

Example 7
RNA isolation

RNA from different human tissues was purchased from Clontech (Human Total RNA Panel I-V). Total RNA from frozen human thyroid tissue was isolated using UltraspecTM-II RNA isolation system (Biotecx Laboratories, Inc.). Total RNA from the TT cell-line (ATCC#CRL1803) was isolated with Trizol reagent (Life Technologies). The RET mutation of five MTC patients had been analyzed: one from a MEN2A family member contained the germline C634R mutation, one patient was positive for the M918T but the germline situation was unknown, and three were sporadic MTCs of which one was positive for the M918T mutation.

Example 8
RT-PCR

The antisense primers used in 5'RACE were mouse specific Gfra4 primer 5'-CAC GTT GTC CAG GTA GTT GG-3' (SEQ ID NO:41:)and a second human specific GFRA4 nested primer 5'-GCA CTG CGC CAC ATA CTC GGA-3' (SEQ ID NO:42:). The 3'region of GFRA4 was cloned in two steps, first using a GFRA4 specific sense primer P2 (see Figure 1A) 5'-GCT CCG AGT ATG TGG CGC AGT-3' (SEQ ID NO:43:) and a nested primer 5'-GCT CAC CCA CGC ACT GCT CTT CTG-3' (SEQ ID NO:44:). The 3'region containing the stop codon

and 3'UTR sequence was cloned using a sense primer 5'-CCT AAC TAC GTG GAC AAC GTG AGC-3' (SEQ ID NO:45:) and a second nested primer 5'-ATG GTG CCA TTC AGG CCT TTG CCA G-3' (SEQ ID NO:46:) or 5'-GCA GGT GTC CTC CAC AGG CAG-3' (SEQ ID NO:47:). RT-PCR of genomic human DNA was used to locate the intronic nucleotide sequences between exons 4 and 5, and between exons 5 and 6, using a sense primer 5'-CCT AAC TAC GTG GAC AAC GTG AGC-3' (SEQ ID NO:45:) and an antisense primer 5'-GAA GTA TGG AGA GCA GGG AGC GTC-3' (SEQ ID NO:48:). Full-length human GFRA4 cDNAs were obtained using a sense primer P1 (see Figure 9A); 5'-CCA CCA TGG TCC GCT GCC TGG-3' (SEQ ID NO:49:) and an antisense primer P3; 5'-GAG GTC GCT GTC CTA ATC AGA G-3' (SEQ ID NO:50:).

Primers used in RT-PCR for human, mouse and rat PSPN were as described in (Milbrandt, et al., 1998). Primers used in RT-PCR for human GFRA1 flanked nucleotides 491-870 of GenBank sequence AF042080, GFRA2 flanked nucleotides 148-427 of GenBank sequence U93703, GFRA3 flanked nucleotides 574-1203 of GenBank sequence NM001496 and RET flanked nucleotides 833-1114 of GenBank sequence X12949. The amounts of total RNA in samples were normalized by amplification of a PPIA fragment.

Example 9
^{125}I-labelled PSPN Binding

Murine neuroblastoma Neuro-2a cells were transfected (FuGene6, Roche) with full-length GFRA4a cDNA in pCR3.1 vector (Invitrogen), and bulk-selected with 400 (g/ml of G418. PSPN was enzymatically iodinated by lactoperoxidase, up to a specific activity of 100.000 cpm/ng. Binding assays were performed essentially as described (Laurikainen, et al., 2000) with 0.9 nM [^{125}I] PSPN in binding buffer (DMEM containing 0.2% BSA and 15 mM Hepes, pH 7.5) for 4 hrs on ice, either in the presence or absence of different concentrations of unlabeled human PSPN (PeproTech EC Ltd.), rat GDNF (Cephalon, Inc.), human NRTN (PeproTech EC Ltd.) or human ARTN (a gift

from Drs. J. Milbrandt and E.M. Johnson, Jr.). The amount of [^{125}I]PSPN bound to mock-transfected cells was at the background level. KD was calculated using Cheng-Prusoff equation (McGonigle, et al., 1994).

Example 10
Chemical cross-linking and immunoprecipitation

CHO cells were transfected (FuGene 6) with full-length human GFRA4a cDNA, grown for two days, washed and incubated with 0.9 nM [^{125}I]PSPN in binding buffer (see above) on ice for 4 hrs. After washing the cells were incubated with 1 mM Bis suberate (Pierce) in PBS at RT for 25 min. Following washes, some samples were treated with 0.5 U/ml of PI-PLC (Sigma) at +37 °C for 30 min. Cells were lysed in Laemmli buffer containing ß-mercaptoethanol.

The cross-linking reaction for human GFRA4a and mock transfected (pcDNA3; Invitrogen) Neuro-2a cells was done as described above but the cells were lysed in NP-40 lysis buffer (1X Tris-borate saline, 2 mM EDTA, 1% Nonidet P-40, 1% Triton X-100, 1 mM PMSF, CompleteTM protease inhibitor cocktail, Roche), clarified by centrifugation at 14 000 rpm for 20 min., immunoprecipitated with 1 (g/ml of Ret antibodies (Santa Cruz Biotechnology) and collected with Protein A Sepharose. The immunocomplexes were washed with NP-40 buffer, and separated by 7.5% SDS-PAGE.

Example 11
Ret Phosphorylation Assay

Semiconfluent human GFRA4a-, rat Gfra1- or vector-expressing Neuro-2a cells were maintained with 0.5% serum for 24 hr and then in serum free medium for 4 hr prior to stimulation. After stimulation with PSPN (0.01-100 ng/ml) or GDNF (100 ng/ml), cells were lysed and precipitated with agarose-conjugated anti-phosphotyrosine antibodies (4G10, Upstate Biotechnology)

overnight at +4 °C on ice. The membranes were probed with 60 ng/ml anti-Ret antibodies (Santa Cruz).

Example 12
Neuronal Microinjections

Postnatal day 1-2 superior cervical ganglion (SCG) neurons were grown 5-6 days on polyornithine-laminin-coated dishes with NGF (30 ng/ml). 50:50 ng/μl cDNA mixtures of short isoform of human RET (gift from Dr. M. Billaud) and either mouse Gfra4a1 (in pcDNA3, Invitrogen) or human GFRA4a (in pCR3.1, not shown) or empty pcDNA3 vector, were pressure-microinjected into nuclei. To find successfully injected neurons later, a plasmid encoding green fluorescent protein (10 ng/μl) was included in every injection mixture. Neurons were grown overnight with NGF and thereafter in NGF-free medium containing blocking anti-NGF antibodies (Boehringer-Mannheim) and PSPN, GDNF, NRTN or ARTN, all at 100 ng/ml. Number of living fluorescent neurons or uninjected control neurons was then counted (initial neurons). 30-80 initial neurons were successfully injected for every treatment group. Healthy fluorescent neurons with intact nuclei and phase-bright cytoplasm were counted 70-75 h later and expressed as percent of initial neurons. Experiments were repeated on independent cultures: n=7 for PSPN, n=4 for GDNF and NRTN, n=3 for ARTN. Significance of the differences between means was estimated by one-way ANOVA followed by Tuckey's post-hoc test at the significance level of α=0.05.

Example 13
Analyzing changes in phosphorylation status

To analyze changes in the phosphorylation status of MAPK (ERK1 and ERK2) and Akt (PKB) in Neuro-2a cell lines stable transfected with either hGFRα4, GFRα1 or empty vector, the semiconfluent cells were kept for 18 hrs in the culture medium containing 0.5% fetal calf serum and then were starved for 3

hours in serum-free medium. After stimulation with GDNF or PSPN, the cells were briefly washed with PBS containing sodium vanadate and sodium fluoride and lysed in NP-40 lysis buffer (Tris-buffered saline; 2 mM EDTA; 1% NP-40; 1% Triton X-100; 1 mM PMSF; 1 mM Na_3VO_4; 25 mM NaF; Complete™ protease inhibitors cocktail (Boehringer Mannheim). The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL (Amersham) membrane. Immunoblotting was carried out using either MAPK (ERK1/2) Anti-Active™ (Promega) or anti-Akt antibodies (New England Biolabs) recognizing phosphorylated forms of the kinases, and detected with ECL system (Amersham). The blots were then reprobed with MAPK or Akt phosphorylation-independent antibodies.

Example 14
Neurite outgrowth assay

To assay neurite outgrowth, cells were plated at 0.5 10^5 cells/cm^2 and maintained in RPMI-1640 containing 1 fetal calf serum supplemented with or without 100 ng/ml either GDNF or PSPN. After 72 hours the percentage of cells with neurites (i.e. bearing processes longer than cell body diameter) was determined. To assay cell survival, cells were washed with serum-free medium, detached and plated in 24-well tissue culture dishes (at approximately 0.5 10^5 cells/cm^2) containing serum-free medium supplemented with either GDNF or PSPN at different concentrations. Cell viability was determined at day 3 after plating, using Trypan Blue dye exclusion method.

The results of the experiments carried out during the studies are disclosed in more detail below.

The Description of the Drawings

Figure 1. Sequence of the predicted GPI-linked mouse GFRα4 protein (SEQ ID NO:2:) and structure of mouse *Gfra4* gene.

Figure 1A. Alignment of mouse GFRα1, GFRα2, GFRα3, and GFRα4 and chicken GFRα4 amino-acid sequences. Predicted signal sequences are underlined. Cysteines are highlighted. The sequence was divided between the predicted hinge regions into four parts and the two cysteins in the homologous Cys-rich domains (D1-D3, see Figure 1D) were aligned manually. The last two amino-acids coded by exons II and exon V are marked by asterisk.

Figure 1B. Phylogenetic tree of the GFRα family. Comparisons were made by using the protein encoding region of mouse *Gfra1-Gfra4* cDNAs.

Figure 1C. Structure of mouse *Gfra4* gene (upper) as compared to mouse *Gfra2* gene (lower) that has alternatively spliced exons 2 and 3 (Baloh et al., 1998). The structures of mouse *Gfra3* (Baloh et al., 1998) and human GFRA1 genes (Angrist et al., 1998) are similar to the mouse *Gfra2* gene, except for an additional short exon that encodes for hinge region in GFRA1 and lack of exon 8 in *Gfra3*. The intron separating exons II and III is very short (53 bp) in *Gfra4* as compared to this intron in other *Gfra* genes (>2 kb). Exon grey shades refer to the homologous Cys-rich domains (D1-D3) and hinge regions in the domain model. N- and C-terminal signal sequences are lined. The lengths of the 5' UTR sequences of exons Ia and Ib (broken line) were not determined.

Figure 1D. Proposed domain structure of mouse GFRα4 (left) and GFRα2 receptor (right) (Airaksinen et al., 1999). Mouse GFRα4 lacks the first domain (D1), which is present in other GFRα-receptors. Putative N-glycosylation sites are also indicated. (>-)

Figure 2. Expressions and developmentally regulated tissue-specific splicing of *Gfra4* gene.

Figure 2A. Northern blot analysis of Gfra4 expression in various mouse tissues (upper). The major transcript in testis appears smaller (appr. 1.4 kb). Longer transcripts (appr. 1.6 kb and 2,5 kb) are also seen. Rehybridization with GAPDH probe (below) demonstrates equal loding.

Figure 2B. Semiquantitative RT-PCR analysis of *Gfra4* transcripts from various tissues and postnatal stages. Locations of PCR primers are indicated on the right and shown in Figure 2C. *Upper panel*: Transcripts containing exon Ia and the spliced intron between exons II and III (Ia/Δi 703 bp) are produced in adrenal, pituitary and thyroid glands but not in brain or testis. 2nd *upper panel*: mRNAs containing exon Ib (Ib/Δi 676 bp) show a expression pattern similar to those containing exon Ia. *Middle panel*: The short intron between exons II and III is efficiently spliced (Δi 550 bp) in thyroid and pituitary glands but not (i 603bp) in brain and testis. 2nd *lower panel*: 3-week-old thyroid glands express the shorter (GPI, 315 bp) splice form, whereas newborn and 6-week-old thyroid glands, preferably as well as postnatal adrenal and pituitary glands, produce the longer (TM 380 bp) splice form. Note that the testis and brain also express their TM or GPI splice forms, but protein synthesis would produce a putative soluble form due to intron inclusion. *Lower panel*: The lines contain similar levels of control Ppian transcript.

Figure 2C. Structures of different *Gfra4* mRNA splice forms containing exon Ia (a1-a4) and exon Ib (b1-b4). Primers P1-P6 used in Figure 2B are indicated below the gene structure. Asterisks denote stop codons. The exon colours are the same as in Figures 1C and 1D. Transcript a1 (SEQ ID NO:7:) is predicted to encode for the GPI-linked isoform of GFRα4 (SEQ ID NO:7:), whereas a2 (SEQ ID NO:8:) encodes for a putative transmembrane (TM)-anchored receptor (SEQ ID NO:2:). Transcripts a3 and a4 (SEQ ID NO:9:) encode for truncated and secreted molecule (domain-2 only) (SEQ ID NO:3:). Transcripts b1-b4 are similar to the transcripts a1-a4 except for usage of exon Ib,

which encodes for a putative weak N-terminal signal sequence instead of exon Ia. Shown in Figures 2A and 2B are representative results from one of three independent experiments. Abbreviations: ad, adult; pit, pituitary gland.

Figure 3. Intron-exon junctions in the coding region of mouse *Gfra4* gene. Consensus splicing sequences are indicated (upper line). There are more non-canonical nucleotides (marked in bold) in the splicing signals of the alternatively spliced introns. Splice acceptor and donor sites are shaded.

Figure 4. Localization of Gfra4 and *Ret* mRNAs in developing and mature mouse tissues by *in situ* hybridization.

Figure 4A. *Gfra4* is highly expressed in the intermediate lobe (Iml) of E18 pituitary gland (sagittal section), while moderate *Ret* expression is seen in the neurohypophysis (Nh). Ah, adenohypophysis.

Figure 4B. *Gfra4* is highly expressed in the intermediate lobe (Iml) of E18 pituitary gland (sagittal section), while moderate *Ret* expression is seen in the neurohypophysis (Nh). Ah, adenohypophysis.

Figure 4C. The condensing mesenchyme of developing limbs (arrows) shows moderate *Gfra4* expression at E16.

Figure 4D. Many neurons of adult spinal cord (coronal section) show moderate to high *Gfra4* expression.

Figure 4E. In adult brain (horizontal section), the distribution of *Gfra4* and *Ret* mRNAs are not overlapping. *Gfra4* is highest in the hippocampus (Hc). Cb, cerebellum.

Figure 4F. In adult brain (horizontal section), the distribution of *Gfra4* and *Ret* mRNAs are not overlapping. *Gfra4* is highest in the hippocampus (Hc). Cb, cerebellum.

Figure 4G. The circular muscle layer and the myenteric ganglia of the adult colon express *Gfra4*. H & E staining.

Figure 4H. The circular muscle layer and the myenteric ganglia of the adult colon express *Gfra4*. Dark field image.

Figure 4I. Seminiferous tubules in adult testis express *Gfra4*. H & E staining.

Figure 4J. Seminiferous tubules in adult testis express *Gfra4*. Dark field image.

Figure 5. *Gfra4* and *Ret* are co-expressed in mouse thyroid C-cells and adrenal chromaffin cells.

Figure 5A. *In situ* hybridization of adjacent sagittal sections of E12 mouse embryos for *Gfra4* (Figure 5A) and *Ret* (Figure 5C). Arrowheads point to the ultimobranchial body. drg dorsal root ganglia.

Figure 5B. *In situ* hybridization of adjacent sagittal sections of E12 mouse embryos for *Gfra4* (Figure 5A) and *Ret* (Figure 5C). Arrowheads point to the ultimobranchial body. drg dorsal root ganglia.

Figure 5C. *In situ* hybridization of adjacent sagittal sections of E12 mouse embryos for *Gfra4* (Figure 5A) and *Ret* (Figure 5C). Arrowheads point to the ultimobranchial body. drg dorsal root ganglia.

Figure 5D. Adjacent sagittal sections of E18 embryos hybridized for *Gfra4* (Figure 5D) and *Ret* (Figure 5F). Both *Gfra4* and *Ret* are expressed in the C-cells but not in the parathyroid gland (broken line).

Figure 5E. Adjacent sagittal sections of E18 embryos

hybridized for *Gfra4* (Figure 5D) and *Ret* (Figure 5F). Both *Gfra4* and *Ret* are expressed in the C-cells but not in the parathyroid gland (broken line).

Figure 5F. Adjacent sagittal sections of E18 embryos hybridized for *Gfra4* (Figure 5D) and *Ret* (Figure 5F). Both *Gfra4* and *Ret* are expressed in the C-cells but not in the parathyroid gland (broken line).

Figure 5G. In 6-week-old mice, *Gfra4* is highly expressed in the thyroid parafollicular C-cells (arrow), but also in the parathyroid gland (broken line). In contrast, *Ret* expression is detectable only in the C-cells (arrow) but not in the parathyroid gland.

Figure 5H. In 6-week-old mice, *Gfra4* is highly expressed in the thyroid parafollicular C-cells (arrow), but also in the parathyroid gland (broken line). In contrast, *Ret* expression is detectable only in the C-cells (arrow) but not in the parathyroid gland.

Figure 5I. In 6-week-old mice, *Gfra4* is highly expressed in the thyroid parafollicular C-cells (arrow), but also in the parathyroid gland (broken line). In contrast, *Ret* expression is detectable only in the C-cells (arrow) but not in the parathyroid gland.

Figure 5J. *Gfra4* is highly expressed by all cells in adrenal medulla at postnatal day P8, while only a subpopulation of them express *Ret*.

Figure 5K. *Gfra4* is highly expressed by all cells in adrenal medulla at postnatal day P8, while only a subpopulation of them express *Ret*.

Figure 5L. *Gfra4* is highly expressed by all cells in adrenal medulla at postnatal day P8, while only a subpopulation of

them express *Ret*. The bright-field images shown in Figures 5B, 5E, 5H, and 5K correspond to those in 5A, 5F, 5I and 5L, respectively.

Figure 6. *Ret*-deficient mice have less thyroid calcitonin-cells.

Figure 6A. E18 thyroid glands from wild-type (Figure 6A) and *Ret*-deficient (Figure 6B) mice are immunostained for calcitonin. Shown are representative sections containing the highest number of labeled C-cells through the middle part of the thyroid gland. The remaining C-cells in *Ret*-deficient mice appear morphologically normal.

Figure 6B. E18 thyroid glands from wild-type (Figure 6A) and *Ret*-deficient (Figure 6B) mice are immunostained for calcitonin. Shown are representative sections containing the highest number of labeled C-cells through the middle part of the thyroid gland. The remaining C-cells in *Ret*-deficient mice appear morphologically normal.

Figure 7. RT-PCR analysis of GFRA4 expression in human thyroid tumors. Each line represents analysis of a tumor from a different patient. The specific 264 bp PCR product representing GFRA4 (exon 1a) expression is high in all medullary carcinoma samples (3/3) but not detectable in other thyroid tumours tested including, 4 follicular adenoma, 4 follicular carcinoma and 5 papillary carcinoma samples.

Figure 8. Localization of human *GFRA4* mRNA expression in medullary thyroid carcinoma but not in other thyroid tumors. *In situ* hybridization was performed using a human *GFRA4* specific antisense cRNA probe on frozen sections.

Figure 8A. *GFRA4* is highly expressed by virtually all cells in the medullary thyroid carcinoma (MTC) but not in the surrounding connective tissue (dark field image).

Figure 8B. Light-field image shows the Nissl-stained structure of tissue.

Figure 8C. Other thyroid tumors, including follicular adenoma (FTA) (Figures 8C and 8D) and papillary carcinoma (PTC) (Figures 8E and 8F) show no *GFRa4* expression above sense control levels (not shown).

Figure 8D. Other thyroid tumors, including follicular adenoma (FTA) (Figures 8C and 8D) and papillary carcinoma (PTC) (Figures 8E and 8F) show no *GFRa4* expression above sense control levels (not shown).

Figure 8E. Other thyroid tumors, including follicular adenoma (FTA) (Figures 8C and 8D) and papillary carcinoma (PTC) (Figures 8E and 8F) show no *GFRa4* expression above sense control levels (not shown).

Figure 8F. Other thyroid tumors, including follicular adenoma (FTA) (Figure 8C and 8D) and papillary carcinoma (PTC) (Figures 8E and 8F) show no *GFRa4* expression above sense control levels (not shown).

Figure 9. Structure of the human *GFRA4* gene compared to mouse *Gfra4* and different splice forms of human *GFRA4* mRNA.

Figure 9A. Structure of the human *GFRA4* gene (top) compared to mouse *Gfra4* gene (bottom). Both contain at least 6 exons but no alternative exon 1b has been found in human. Also the putative transmembrane form encoded by the alternatively spliced exon 6 found in mouse is absent in human. Primers P1-P3, used in RT-PCR in Figures 2 and 5 are marked by arrowheads.

Figure 9B. Splicing of *GFRA4* gene in human thyroid gland. Two GPI-linked isoforms (*GFRA4a* and *GFRA4b*), and one putative

soluble isoform (*GFRA4c*) are produced by alternative splicing. In transcript encoding for GFRα4a all the introns are spliced, whereas GFRα4b contains the intron between exon 2 and exon 3. Transcript encoding GFRα4c contain introns between exons 2 and 3 and between exons 3 and 4. Asterisk marks the stop codon.

Figure 9C. Proposed schematic domain structure of human GFRα4 isoforms.

Figure 10. RT-PCR analysis of *GFRA4, PSPN* and *RET* transcripts in various human tissues. Top row: *GFRA4* expression is detected only in thyroid gland using primers P2 and P3 (see Figure 15A). All the introns in the transcript corresponding to 699 bp are spliced. The 789 bp transcript contains a small intron between exon 2 and exon 3 and corresponds to the GFRα4b form. The 863 bp fragment contains introns between exons 2 and 3 and between exons 3 and 4, and corresponds to the soluble GFRα4c form. Second row: An unspliced transcript of the human *PSPN* is expressed in all tissues (approximately 590 bp, intron size not determined), whereas the spliced transcript (476 bp), which corresponds to the functional PSPN, is weakly expressed in cerebellum, adrenal gland and testis and barely detectable in spinal cord. To exclude DNA contamination in RT-PCR reactions, controls containing RNA were also subjected to PCR (not shown). Third row: *RET* expression (282 bp) is detectable in almost all human tissues examined but the expression levels are strongest in adult brain, cerebellum, spinal cord, salivary gland, adrenal gland and prostate. Bottom row: Control RT-PCR with *PPIA* transcript showing equal loading of cDNA. In all PCR experiments, negative water controls were included (not shown).

Figure 11. Binding and Chemical Cross-linking of [^{125}I]PSPN to Human GFRα4 and Ret.

Figure 11A. Displacement binding of [^{125}I]PSPN by PSPN, NRTN, GDNF and ARTN from human GFRα4a-expressing Neuro-2a cells,

shown as percentage of [^{125}I]PSPN bound in the absence of a cold ligand (100%). One representative experiment out of four is shown.

Figure 11B. Chemical cross-linking of [^{125}I]PSPN to vector- (lanes 1, 2) or h*GFRA4a* transfected (lanes 3-6) CHO cells, in the presence (lanes 2, 3, 5) or absence (lanes 1, 4, 6) of unlabeled PSPN, treated (lanes 3, 4) or not treated (lanes 1, 2, 5, 6) with PI-PLC. Bands are designated as follows: arrowhead, approximately 46 kD; arrow, approximately 62 kD; asterisk, approximately 92 kD.

Figure 11C. Proteins cross-linked to [^{125}I]PSPN in the presence (lanes 1, 3) or absence (lanes 2, 4) of unlabeled PSPN in vector- (lanes 1, 2) or *GFRA4a*- (lanes 3, 4) transfected Neuro-2a cells. Bands are designated as in Figure 11B.

Figure 11D. Immunoprecipitation of Ret from vector- (lanes 1, 3) or *GFRA4a*-transfected (lanes 2, 4) Neuro-2a cells after chemical cross-linking of [^{125}I]PSPN, separated in the presence (lanes 1, 2) or absence (lanes 3, 4) of (ß-mercaptoethanol (ß-Me). Bands are designated as follows: arrowhead, approximately 46 kD; arrow, approximately 196 kD; diamond approximately 232 kD; asterisk, approximately 400 kD.

Figure 11E. Phosphorylation of Ret in mock- (lanes 1, 2), *GFRA4a*- (lanes 3, 4, 5) or *Gfra1*-transfected Neuro-2a cells upon stimulation with 100 ng/ml of PSPN (lanes 2, 4, 5) or 100 ng/ml of GDNF (lane 7).

Figure 11F. Dose-dependent phosphorylation of Ret with PSPN in h*GFRA4a*-transfected Neuro-2a cells.

Figure 12. PSPN specifically promotes survival of *RET*/*Gfra4*-injected SCG neurons. Neonatal mouse SCG neurons

were grown five days with NGF, injected with indicated plasmid mixtures (all 50:50 ng/μl), grown overnight with NGF, then NGF was changed for indicated factors (all 100 ng/ml). Numbers of viable neurons after 3-day treatment period are expressed relative to the number of initial neurons. Means ± s.e.m of three to seven independent repeats for each factor is shown. $p<0.05$ (*); $p<0.001$ (***).

Figure 13. Semiquantitative RT-PCR analysis of *GFRA4*, *PSPN* and *RET* in different human thyroid tumors. Top row: *GFRA4* mRNA is selectively expressed in medullary thyroid carcinomas (MTC). No *GFRA4* transcripts are detectable in follicular thyroid adenomas (FTA), follicular thyroid carcinomas (FTC) or papillary thyroid carcinomas (PTC). Each lane represents tumor cDNA from different patient. Normal thyroid gland, including medullary tissue (NT) also expresses *GFRA4*. Two major transcripts of 831 and 910 bp seen in MTC and TT-cells correspond to GFRα4a and GFRα4b, respectively (primers P1 and P3, see Figure 15A). The minor transcript of 999 bp corresponds to the soluble GFRα4c. Second row: No spliced form of the *PSPN* transcript is detectable using primers covering the initiation and the stop codons. Third row: *RET* mRNA is expressed in all MTC samples. Bottom row: Control RT-PCR with *PPIA* transcript showing equal loading of cDNA. All experiments contained negative water controls (not shown).

Figure 14. Localization of *GFRA1*, *GFRA2* *GFRA3*, *GFRA4* and *RET* mRNAs in thyroid tumors by *in situ* hybridization.

Figure 14A. Typical representative examples of autoradiography films showing *GFRA1-GFRA4* and *RET* cRNA hybridization to adjacent frozen sections of different thyroid tumors. Of the four types of tumors represented, only the MTC sections were strongly stained for *GFRA4* and *RET* expression. *GFRA1*, *GFRA2*, *GFRA3* mRNA levels were very low or undetectable in all samples including MTC.

Figure 14B. *GFRA4* and *RET* probes were hybridized to frozen sections representing follicular thyroid adenoma (FTA), follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC), and medullary thyroid carcinoma (MTC) samples and sections were counter-stained by Nissl-substance. *GFRA4* and *RET* is highly expressed in all malignant cells of the MTC sample but not in the surrounding connective tissue. Bar in A= 0.5 cm, in B=100 μm.

Figure 15. Activation of Akt in Neuro-2a cells expressing either GFRα1 or hGFRα4 with their respective ligands.

Figure 15A. Neither GDNF nor PSPN (native or heat-inactivated) stimulate Akt phosphorylation in mock-transfected Neuro-2a cells. In Neuro-2a cells expressing GFRα1, GDNF, but not PSPN evokes Akt phosphorylation.

Figure 15B. PSPN, but not GDNF, stimulates Akt phosphorylation in Neuro-2a cells expressing GFRα4. Upper panels in Figure 15A and Figure 15B show immunoblotting of the cell lysates with anti phospho-Akt (Ser 473) antibodies. Lower panels show the re-probing of the same blots with phosphorylation state-independent anti Akt antibodies.

Figure 16. MAP kinases are activated in response to GDNF but not to PSPN in the Neuro-2a cells.

Figure 16A. Neither GDNF, nor PSPN (native or heat-inactivated), stimulate MAPK phosphorylation in mock-transfected Neuro-2a cells. In Neuro-2a/GFRα1 cells GDNF, but not PSPN, activates MAPK.

Figure 16B. In Neuro-2a/hGFRα4 cells PSPN is unable to stimulate phosphorylation of MAPK. Upper panels in Figure 16A and Figure 16B show immunoblotting of the cell lysates with anti phospho-MAPK antibodies. Lower panels show the re-probing

of the same blots with phosphorylation state-independent anti MAPK antibodies.

Figure 17A. GDNF and PSPN both stimulate cell survival in dose-dependent manner via their respective receptors in serum-free conditions.

Figure 17B. PSPN, in contrast to GDNF, cannot induce neurite outgrowth in Neuro-2a/hGFRα4 cells. Shown are mean values with SEM. ** - $P<0.01$; *** - $P<0.001$.

Figure 18. cDNA and amino-acid sequences of mouse GFRα4 GPI-isoform a1. The GPI-linked isoform, (cf. Figure 1A) corresponds to transcript a1 in Figure 2C. The signal sequence encoded by exon 1a is underlined.

Figure 19. The putative TM-anchored isoform, GFRα4-a2, encoded by transcript a2 in Figure 2C.

Figure 20. cDNA and amino-acid sequence of GFRα4-a3/4, a soluble isoform of mouse GFRα4. The signal sequence encoded by exon 1a is underlined. GFRα4-a3/4 is encoded by transcripts a3-a4 as shown in Figure 2C.

Figure 21. Shown are the cDNA and amino-acid sequence of human GFRα4 a GPI-linked isoform a.

Figure 22. Human GFRα4 b, putative GPI-linked isoform b.

Figure 23. Human GFRα4 c, a putative secreted isoform, formed by alternative splicing of the second short intron.

Figure 24. Genomic sequence of human GFRA4 locus. The coding sequences are in uppercase. Intronic sequences are in lowercase and bold. The alternatively spliced 3'-end of the 2nd short intron is shown in italics.

Table 1

Expression of *Gfra4* and *Ret* mRNAs in Selected Mouse Tissues and Cells

Age	E12		E16		P0		P20		adult	
Area	*Gfra4*	*Ret*	*Gfra4*	*Ret*	*Gfra*	*Ret*	*Gfra4*	*Ret*	*Gfra4*	*Ret*
NC	±	-	++	-	++	-	nd	nd	++	-
HC	nd	nd	nd	nd	++	-	nd	nd	+++	+
VM	+	+++	nd	nd	+	+++	nd	nd	+	+++
VSC	+	+++	++	+++	+	+++	nd	nd	+	+++
PNS	±	+++	+	+++	++	+++	++	+++	++	+++
PI	nd	nd	+++	-	+++	-	+++	nd	+++	-
TC	++	++	+++	++	+++	++	+++	++	+++	+
PT	nd	nd	-	-	±	-	+++	-	+++	-
AdM	nd	nd	nd	nd	++	++*	++	+	++	+*
Kidney	+	+++	+	+++	±	+++	-	+	-	±
Lung	+	+	+	+	±	-	nd	nd	-	-
Liver	-	-	-	-	-	-	nd	nd	-	-
Testis	nd	nd	nd	nd	+	++*	++	±	++	±

mRNA expression was graded from -, no labeling above background, to +++, highest detected expression level based on visual inspection of *in situ* hybridization sections. Expression of Gfra4 and Ret at E14 and E16 as well as P8 and P20 were overlapping. *Expression differs from that reported by Golden et al., 1999.

Abbreviations

IML, intermediate lobe; nd, not determined; PNS, peripheral nervous system including sensory (DRG), sympathetic (SGG) and enteric ganglia; NC, Neocortex; HC, Hippocampus; VM, Ventral Midbrain; VSC, Ventral Spinal Cord; PNS, PNS ganglia; PI, Pituitary (IML); TC, Thyroid (C-cells); PT, Parathyroid; AdM, Adrenal Medulla; nd, not determinated.

Table 2

Expression of human *GFRA1, GFRA2, GFRA3, GFRA4* and *RET* in thyroid tumours and normal thyroid

Tumour	*GFRA1*		*GFRA2*		*GFRA3*		*GFRA4*		*RET*	
	RT	*In situ*	RT	*In Situ*	RT	*In situ*	RT	*In situ*	RT	*In situ*
FTA	+ 1/4	+ 1/2	+ 3/4	+ 2/2	+ 4/4	+ 2/2	- 0/4	- 0/3	- 0/4	- 0/2
FTC	+ 1/4	- 0/1	+ 2/4	+ 1/1	+ 2/4	+ 1/2	- 0/4	- 0/3	- 0/4	- 0/1
PTC	+ 2/5	- 0/2	+ 3/5	+ 2/2	+ 2/5	+ 1/3	- 0/5	- 0/4	- 0/5	- 0/2
MTC	+ 5/8	+ 4/6	+ 7/8	+ 4/6	+ 4/8	+ 2/6	+++ 8/8	+++ 6/6	+++ 8/8	+++ 6/6
NT	+ 1/1	Nd	+ 1/1	Nd	+ 2/3	Nd	++ 1/3	Nd	++ 1/1	Nd
TT cells	+	Nd	-	Nd	-	Nd	+++	Nd	+++	Nd

mRNA expression is graded from -, no labelling above background, to +++, highest detected expression based on visual inspection. The numbers below the tissue gradings denote the number of samples positive/total number of samples analyzed.

Abbreviations

RT, RT-PCR; FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; MTC, medullary thyroid carcinoma; NT, normal thyroid; Nd, not determined.

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What is claimed:

1. An isolated and purified nucleic acid sequence, characterized in that the nucleic acid sequence is a cDNA encoding a splicing isoform of mammalian GFRα4 comprising the amino acid sequence (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:), (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) or fragments thereof.

2. The nucleic acid sequence according to claim 1, characterized in that the nucleic acid sequence is a cDNA encoding a splicing isoform of murine GFRα4 comprising the amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) or fragments thereof.

3. The nucleic acid sequence according to claim 1, characterized in that the nucleic acid sequence is a cDNA encoding a splicing isoform of human GFRα4 comprising of the amino acid sequence (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) or fragments thereof.

4. The nucleic acid sequence according to claim 1, characterized in that the nucleic acid sequence is a cDNA having the sequence (SEQ ID NO:7:), (SEQ ID NO:8:), (SEQ ID NO:9:), (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:) or fragments thereof, encoding a polypeptide having substantially similar functions as human or mouse GFRα4.

5. The nucleic acid sequence according to claim 1, characterized in that the nucleic acid sequence is a cDNA encoding a human GFRα4 characterized by comprising the nucleic acid sequence (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:), said (SEQ ID NO:13:) being a copy of the genomic sequence without or with introns or fragments of the introns with the prerequisite that it includes at least one of the exons or parts thereof (SEQ ID

NO:29:), (SEQ ID NO:30:), (SEQ ID NO:31:), (SEQ ID NO:33:), (SEQ ID NO:35:), (SEQ ID NO:35:), (SEQ ID NO:37:) or (SEQ ID NO:39:), fragments or any combination thereof having fragments of the introns (SEQ ID NO:32:), (SEQ ID NO:34:), (SEQ ID NO:36) or (SEQ ID NO:38) but still encoding a polypeptide having a functional activity substantially similar to that of human GFRα4.

6. The use of nucleic acid sequence according to claims 1-5 for recording GFRα4-mediated signalling in neurons or endocrine cells.

7. The use of nucleic acid sequence according to claims 6, wherein the endocrine cells are thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells or cells of pituitary intermediate lobe.

8. The use of nucleic acid sequence according to claim 1-5 for manufacturing polypeptides useful for diagnosing and/or treating tumours in parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma and pheochromocytoma, parathyroid hyperplasia, neuronal disorders or for preventing neuronal death or aberrant axonal sprouting.

9. A purified and isolated polypeptide, characterized in that it comprises a polypeptide having the amino acid sequence (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:), (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:).

10. The polypeptide according to claim 9, characterized in that it has a structure substantially similar to an isoform of mouse GFRα4.

11. The polypeptide according to claim 9, characterized in that it has a structure substantially similar to an isoform of human GFRα4.

12. The use of the polypeptides according to claims 9-11 for determining GFRα4-mediated signalling in neurons, endocrine cells, neoplasia, endocrine tumours, medullary thyroid carcinoma.

13. The use of the polypeptides according to claims 12, wherein the endocrine cells are thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells or cells of pituitary intermediate lobe.

14. The use of the polypeptides according to claims 12, wherein the medullary thyroid carcinoma comprises pheochromocytoma and parathyroid hyperplasia.

15. The use of polypeptides according to claims 9-11 for manufacturing a composition for diagnosing and/or treating neuronal disorders, osteoporosis, cancer in parathyroid gland cells, adrenal chromaffin cells and cells of pituitary intermediate lobe, in neoplasia, treatment of endocrine tumours, including medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia, for preventing axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures, preventing neuronal death without producing aberrant sprouting.

16. A method for obtaining a cell-line (clone) useful in the production of the transgenic non-human animal according to claims, c h a r a c t e r i z e d in that the genomic gene encoding a functional GFRα4 receptor is inactivated by introducing into stem cells or cell lines (clones) a nucleic acid sequence or a part thereof, which sequence or part is capable of integrating to said genomic gene and by said integration functionally inactivates said genomic gene or makes it conditionally inactivatable.

17. A cell line (clone) obtainable by the method of claim 16,

c h a r a c t e r i z e d in that the cell-line (clone) is lacking a functional GFRα4 receptor and producing altered levels of GFRα4.

18. The use of the cell line (clone) of claim 17, c h a r a c t e r i z e d in that the cell lines are used for producing a transgenic non-human animal and/or diagnosing and/or treating osteoporosis, endocrine tumours, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia by recording GFRα4-mediated signalling in endocrine cells, thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia, neuronal disorders, for preventing axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures or preventing neuronal death without producing aberrant sprouting.

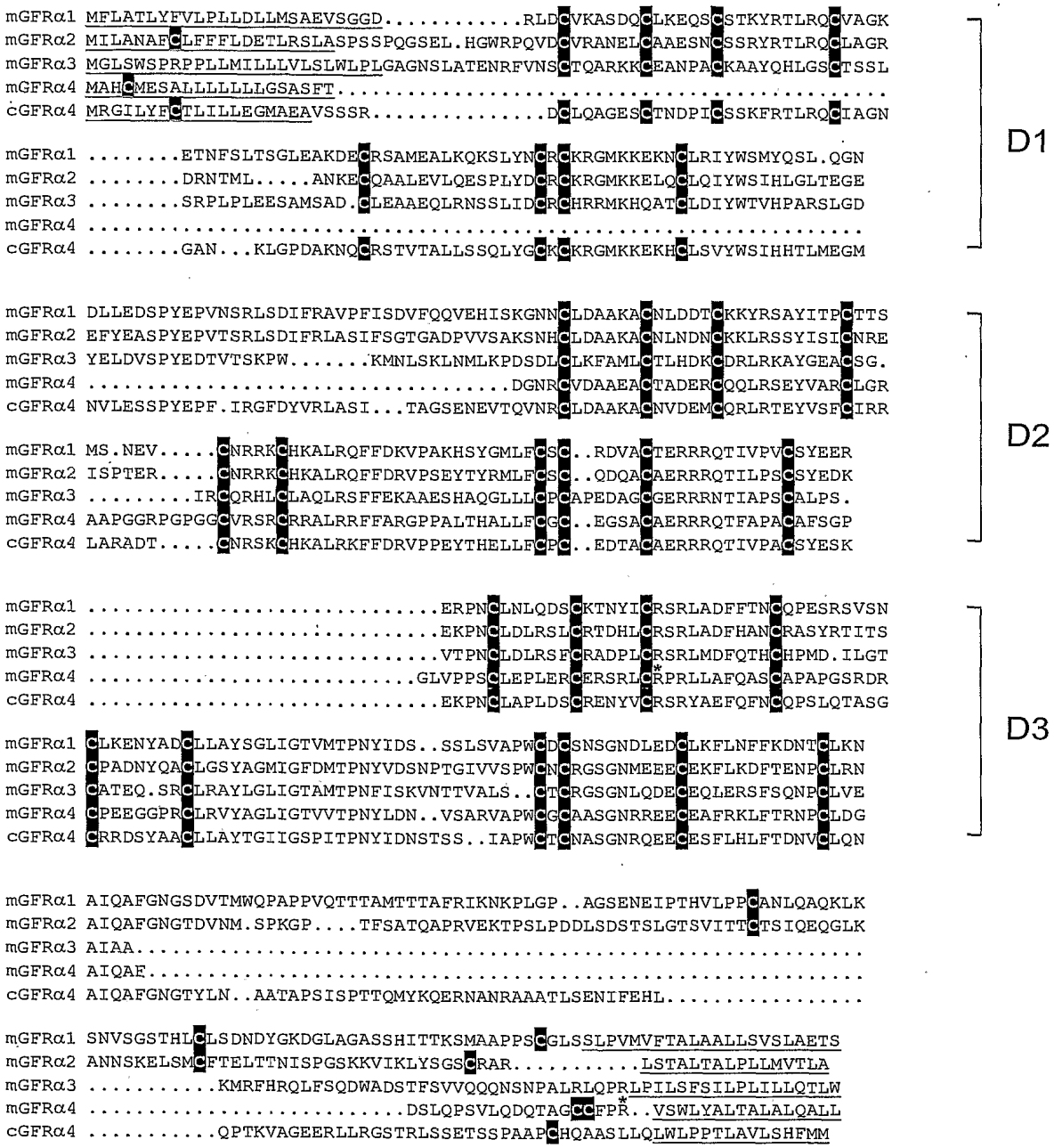
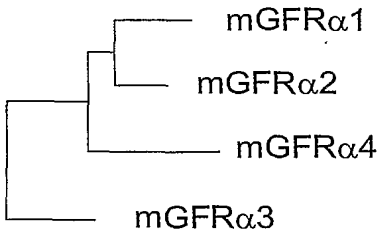
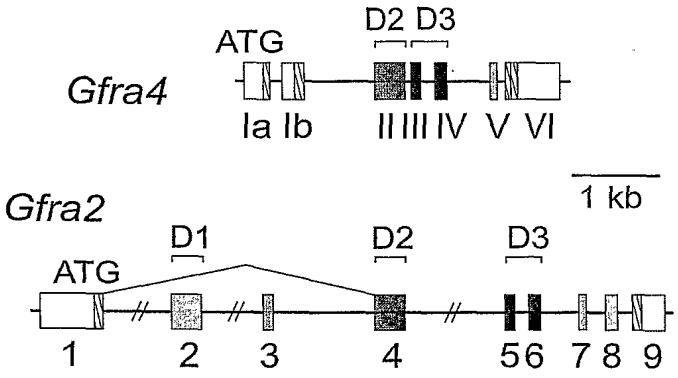

mGFRα1 MFLATLYFVLPLLDLLMSAEVSGGD.....RLDCVKASDQCLKEQSCSTKYRTLRQCVAGK
mGFRα2 MILANAFCLFFFLDETLRSLASPSSPQGSEL.HGWRPQVDCVRANELCAAESNCSSRYRTLRQCLAGR
mGFRα3 MGLSWSPRPPLLMILLLVLSLWLPLGAGNSLATENRFVNSCTQARKKCEANPACKAAYQHLGSCTSSL
mGFRα4 MAHCMESALLLLLLLGSASFT.....
cGFRα4 MRGILYFCTLILLEGMAEAVSSSR.....DCLQAGESCTNDPICSSKFRTLRQCIAGN
mGFRα1 ........ETNFSLTSGLEAKDECRSAMEALKQKSLYNCRCKRGMKKEKNCLRIYWSMYQSL.QGN
mGFRα2 ....DRNTML.....ANKECQAALEVLQESPLYDCRCKRGMKKELQCLQIYWSIHLGLTEGE
mGFRα3 ........SRPLPLEESAMSAD.CLEAAEQLRNSSLIDCRCHRRMKHQATCLDIYWTVHPARSLGD
mGFRα4 .....
cGFRα4 ....GAN...KLGPDAKNQCRSTVTALLSSQLYGCKCKRGMKKEKHCLSVYWSIHHTLMEGM
D1
mGFRα1 DLLEDSPYEPVNSRLSDIFRAVPFISDVFQQVEHISKGNNCLDAAKACNLDDTCKKYRSAYITPCTTS
mGFRα2 EFYEASPYEPVTSRLSDIFRLASIFSGTGADPVVSAKSNHCLDAAKACNLNDNCKKLRSSYISICNRE
mGFRα3 YELDVSPYEDTVTSKPW.....KMNLSKLNMLKPDSDLCLKFAMLCTLHDKCDRLRKAYGEACSG.
mGFRα4 .....DGNRCVDAAEACTADERCQQLRSEYVARCLGR
cGFRα4 NVLESSPYEPF.IRGFDYVRLASI...TAGSENEVTQVNRCLDAAKACNVDEMCQRLRTEYVSFCIRR
mGFRα1 MS.NEV.....CNRRKCHKALRQFFDKVPAKHSYGMLFCSC..RDVACTERRRQTIVPVCSYEER
mGFRα2 ISPTER.....CNRRKCHKALRQFFDRVPSEYTYRMLFCSC..QDQACAERRRQTILPSCSYEDK
mGFRα3 .....IRCQRHLCLAQLRSFFEKAAESHAQGLLLCPCAPEDAGCGERRRNTIAPSCALPS.
mGFRα4 AAPGGRPGPGGCVRSRCRRALRRFFARGPPALTHALLFCGC..EGSACAERRRQTFAPACAFSGP
cGFRα4 LARADT.....CNRSKCHKALRKFFDRVPPEYTHELLFCPC..EDTACAERRRQTIVPACSYESK
D2
mGFRα1 .....ERPNCLNLQDSCKTNYICRSRLADFFTNCQPESRSVSN
mGFRα2 .....EKPNCLDLRSLCRTDHLCRSRLADFHANCRASYRTITS
mGFRα3 .....VTPNCLDLRSFCRADPLCRSRLMDFQTHCHPMD.ILGT
mGFRα4 .....GLVPPSCLEPLERCERSRLCRPRLLAFQASCAPAPGSRDR
cGFRα4 .....EKPNCLAPLDSCRENYVCRSRYAEFQFNCQPSLQTASG
mGFRα1 CLKENYADCLLAYSGLIGTVMTPNYIDS..SSLSVAPWCDCSNSGNDLEDCLKFLNFFKDNTCLKN
mGFRα2 CPADNYQACLGSYAGMIGFDMTPNYVDSNPTGIVVSPWCNCRGSGNMEEECEKFLKDFTENPCLRN
mGFRα3 CATEQ.SRCLRAYLGLIGTAMTPNFISKVNTTVALS..CTCRGSGNLQDECEQLERSFSQNPCLVE
mGFRα4 CPEEGGPRCLRVYAGLIGTVVTPNYLDN..VSARVAPWCGCAASGNRREECEAFRKLFTRNPCLDG
cGFRα4 CRRDSYAACLLAYTGIIGSPITPNYIDNSTSS..IAPWCTCNASGNRQEECESFLHLFTDNVCLQN
D3
mGFRα1 AIQAFGNGSDVTMWQPAPPVQTTTAMTTTAFRIKNKPLGP..AGSENEIPTHVLPPCANLQAQKLK
mGFRα2 AIQAFGNGTDVNM.SPKGP....TFSATQAPRVEKTPSLPDDLSDSTSLGTSVITTCTSIQEQGLK
mGFRα3 AIAA.....
mGFRα4 AIQAF.....
cGFRα4 AIQAFGNGTYLN..AATAPSISPTTQMYKQERNANRAAATLSENIFEHL.....
mGFRα1 SNVSGSTHLCLSDNDYGKDGLAGASSHITTKSMAAPPSCGLSSLPVMVFTALAALLSVSLAETS
mGFRα2 ANNSKELSMCFTELTTNISPGSKKVIKLYSGSCRAR.....LSTALTALPLLMVTLA
mGFRα3 .....KMRFHRQLFSQDWADSTFSVVQQQNSNPALRLQPRLPILSFSILPLILLQTLW
mGFRα4 .....DSLQPSVLQDQTAGCCFPR..VSWLYALTALALQALL
cGFRα4 .....QPTKVAGEERLLRGSTRLSSETSSPAAPCHQAASLLQLWLPPTLAVLSHFMM

Fig. 1A

1B



1C



1D

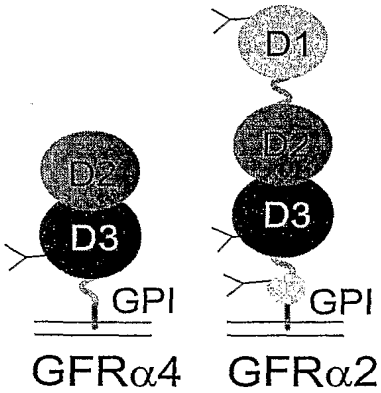


Fig. 1B-D

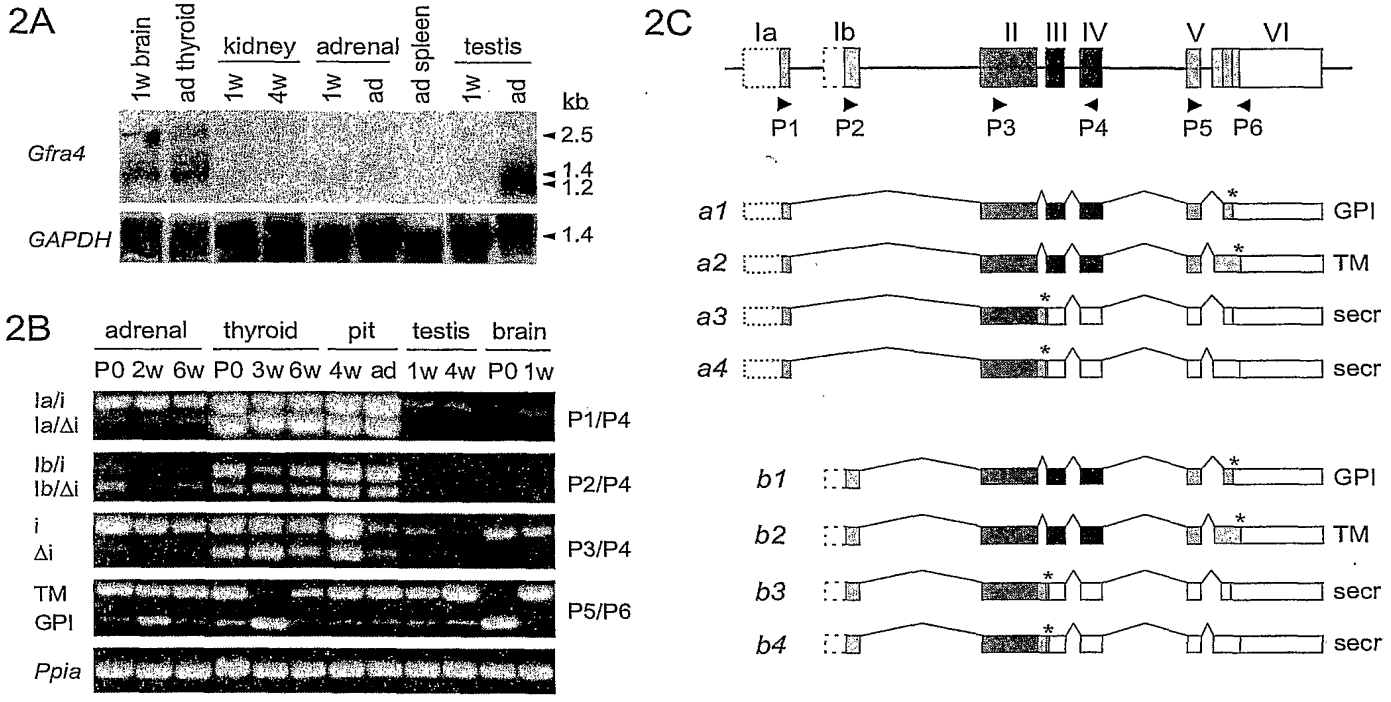

2A
1w brain
ad thyroid
kidney
1w
4w
adrenal
1w
ad
ad spleen
testis
1w
ad
kb
Gfra4
2.5
1.4
1.2
GAPDH
1.4
2B
adrenal
thyroid
pit
testis
brain
P0 2w 6w P0 3w 6w 4w ad 1w 4w P0 1w
Ia/i
Ia/Δi
P1/P4
Ib/i
Ib/Δi
P2/P4
i
Δi
P3/P4
TM
GPI
P5/P6
Ppia
2C
Ia
Ib
II
III
IV
V
VI
P1
P2
P3
P4
P5
P6
a1
GPI
a2
TM
a3
secr
a4
secr
b1
GPI
b2
TM
b3
secr
b4
secr

Fig. 2

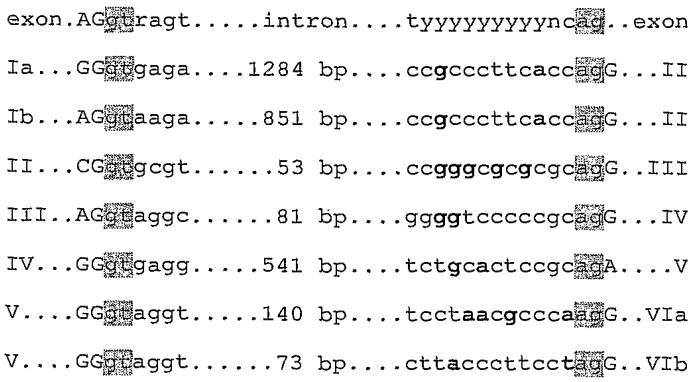

exon.AGgtragt.....intron....tyyyyyyyyyncag..exon
Ia...GGgtgaga....1284 bp....ccgcccttcaccagG...II
Ib...AGgtaaga.....851 bp....ccgcccttcaccagG...II
II...CGgtgcgt......53 bp....ccgggcgcgcgcagG..III
III..AGgtaggc......81 bp....ggggtcccccgcagG...IV
IV...GGgtgagg.....541 bp....tctgcactccgcagA....V
V....GGgtaggt.....140 bp....tcctaacgcccaagG..VIa
V....GGgtaggt......73 bp....cttacccttcctagG..VIb

Fig. 3

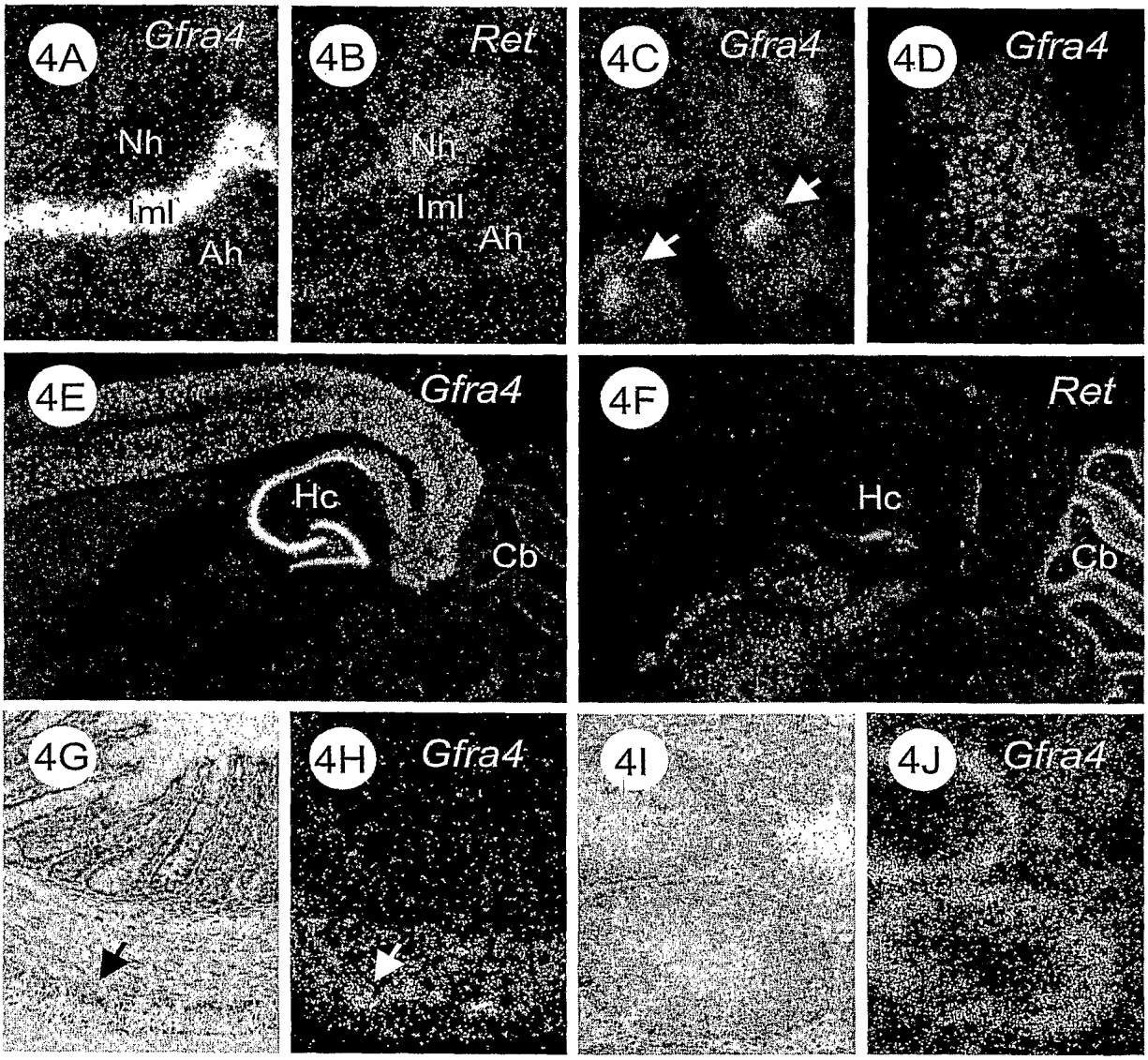

4A
Gfra4
Nh
Iml
Ah
4B
Ret
Nh
Iml
Ah
4C
Gfra4
4D
Gfra4
4E
Gfra4
Hc
Cb
4F
Ret
Hc
Cb
4G
4H
Gfra4
4I
4J
Gfra4

Fig. 4

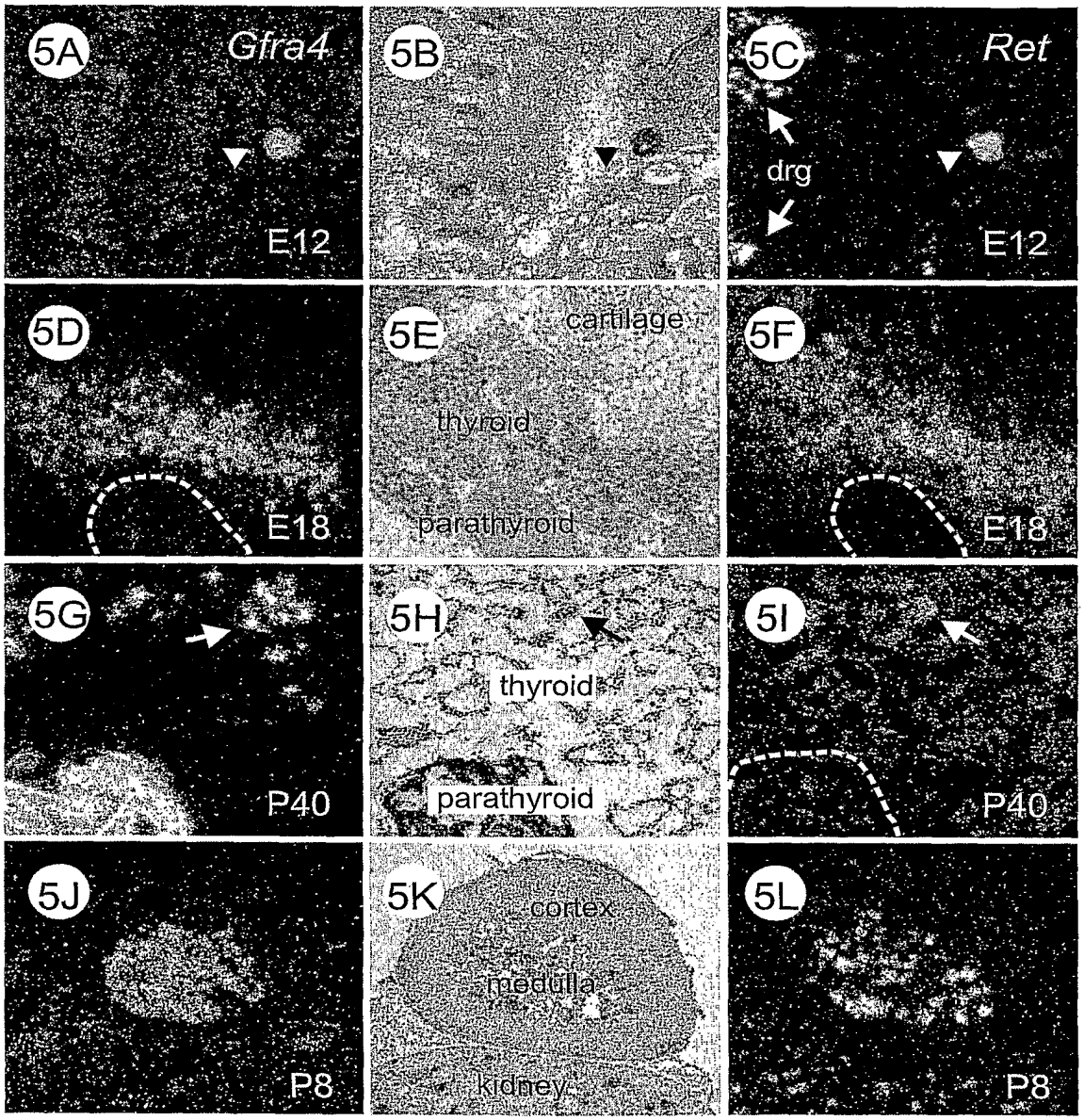

5A
Gfra4
E12
5B
5C
Ret
drg
E12
5D
E18
5E
cartilage
thyroid
parathyroid
5F
E18
5G
P40
5H
thyroid
parathyroid
5I
P40
5J
P8
5K
cortex
medulla
kidney
5L
P8

Fig. 5

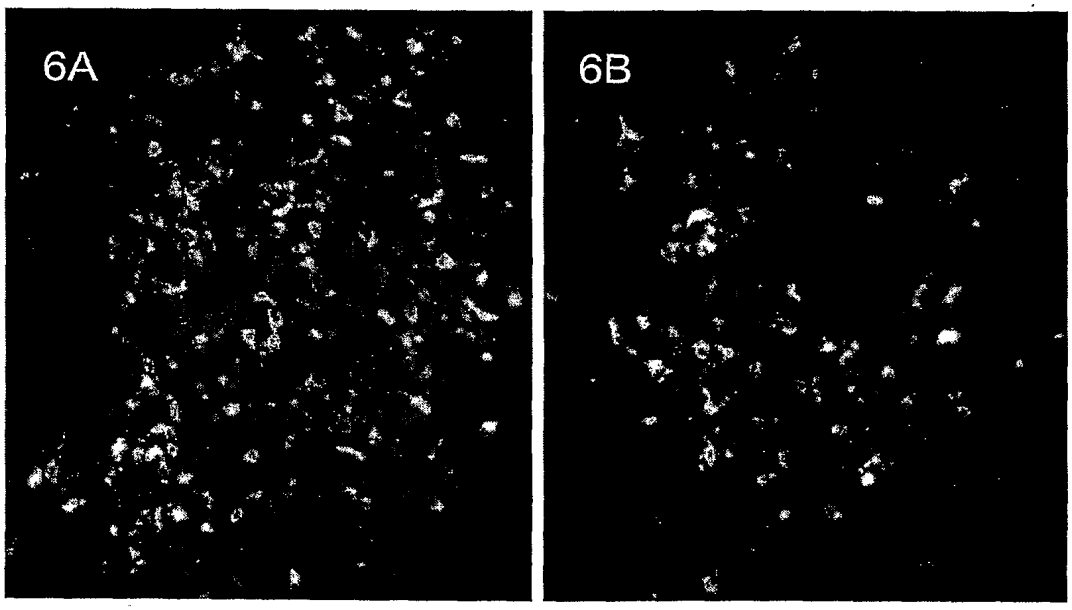


Fig. 6

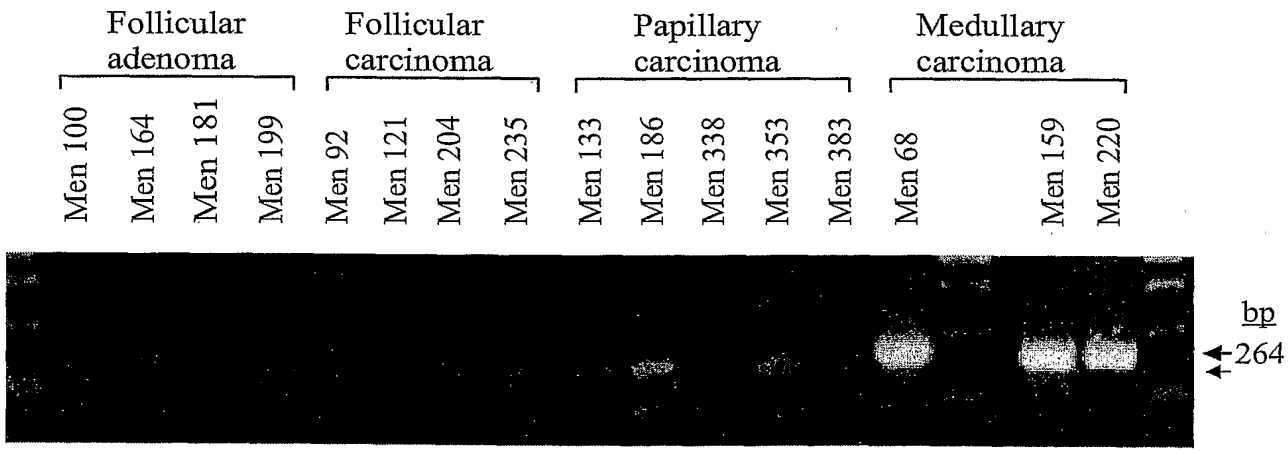

Follicular adenoma
Follicular carcinoma
Papillary carcinoma
Medullary carcinoma
Men 100
Men 164
Men 181
Men 199
Men 92
Men 121
Men 204
Men 235
Men 133
Men 186
Men 338
Men 353
Men 383
Men 68
Men 159
Men 220
bp
264

Fig. 7

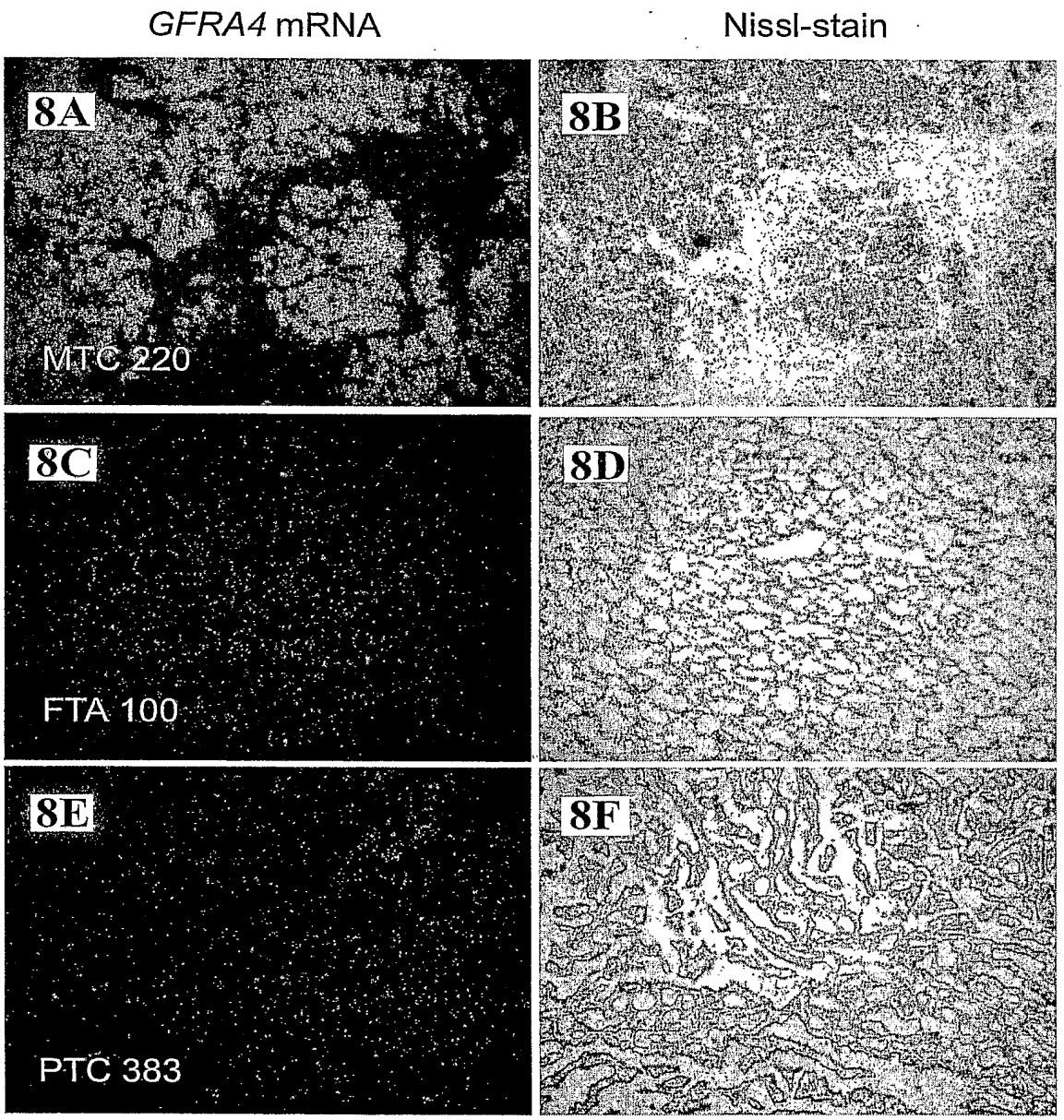
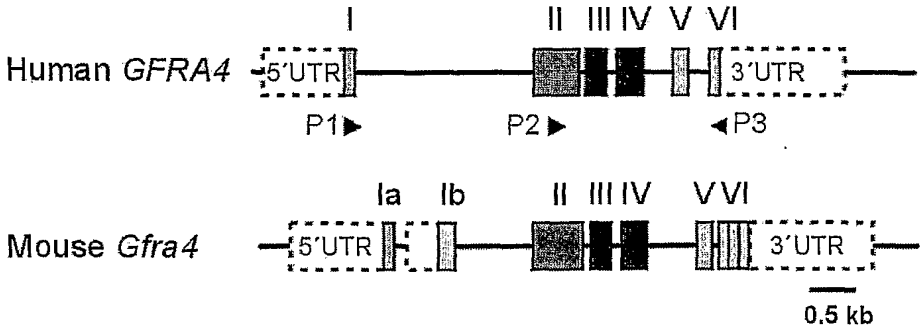
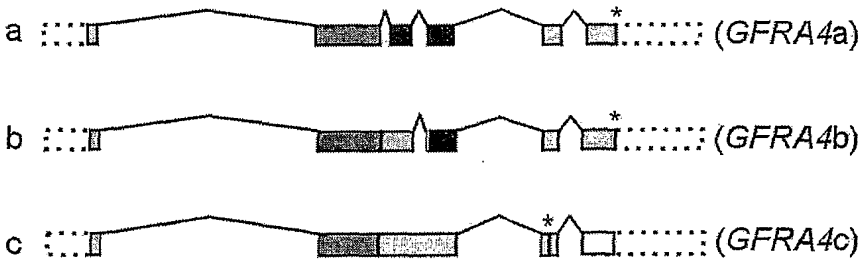


Fig. 8

9 A


Human GFRA4
I
II III IV V VI
5'UTR
3'UTR
P1
P2
P3
Mouse Gfra4
Ia Ib
II III IV V VI
5'UTR
3'UTR
0.5 kb

9 B


a
(GFRA4a)
b
(GFRA4b)
c
(GFRA4c)

9C

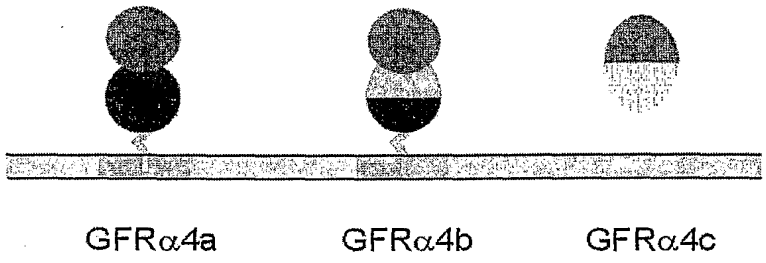

GFRα4a
GFRα4b
GFRα4c

Fig. 9

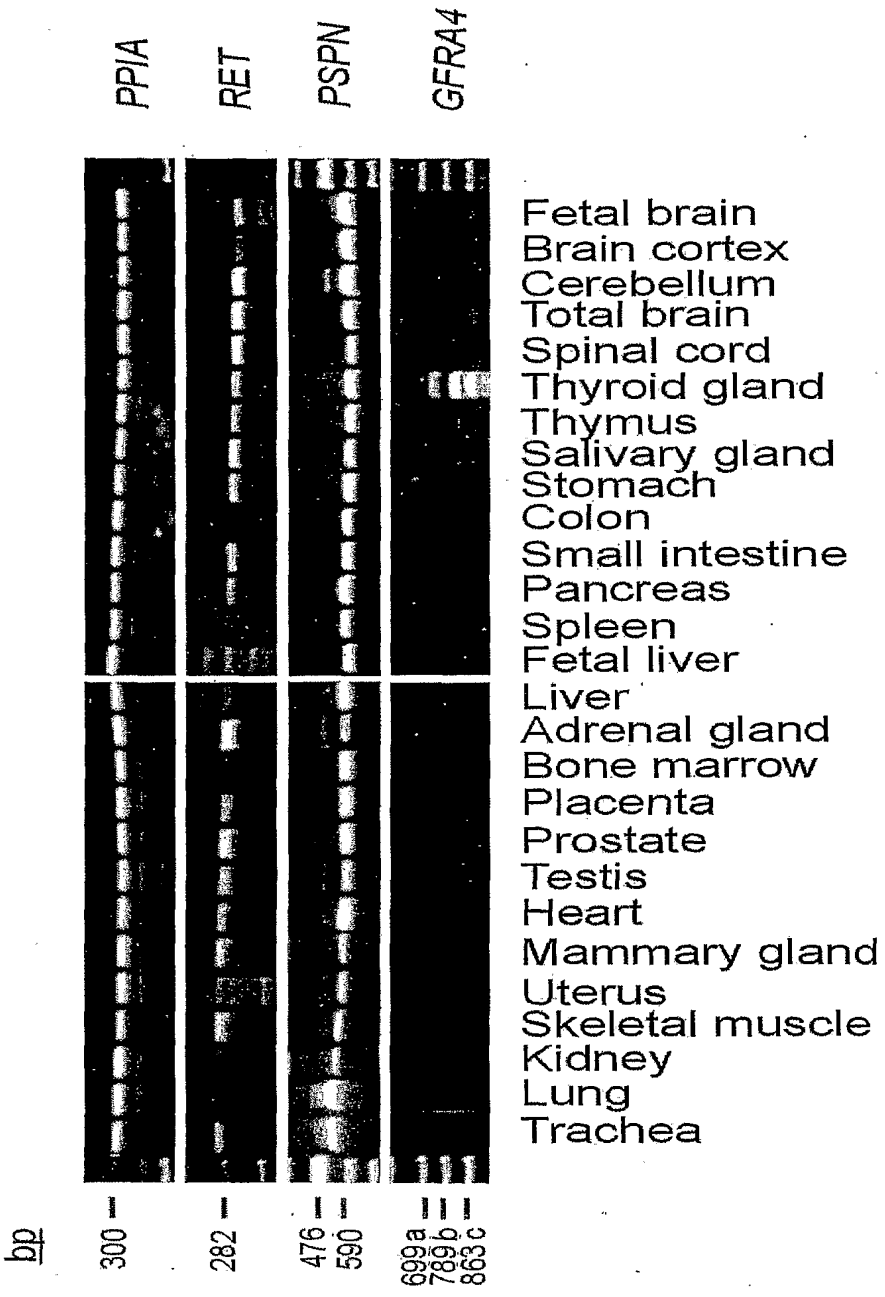

PPIA
RET
PSPN
GFRA4
Fetal brain
Brain cortex
Cerebellum
Total brain
Spinal cord
Thyroid gland
Thymus
Salivary gland
Stomach
Colon
Small intestine
Pancreas
Spleen
Fetal liver
Liver
Adrenal gland
Bone marrow
Placenta
Prostate
Testis
Heart
Mammary gland
Uterus
Skeletal muscle
Kidney
Lung
Trachea
bp
300
282
476
590
699a
789b
863c

Fig. 10

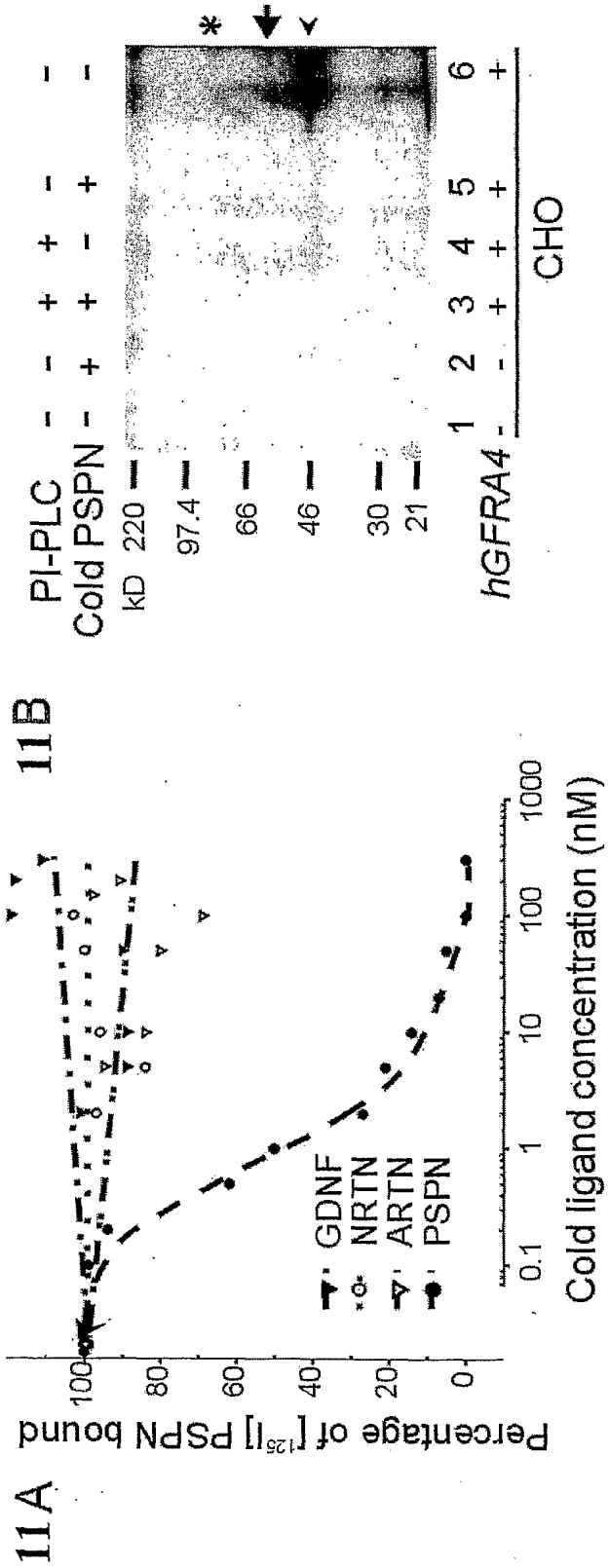
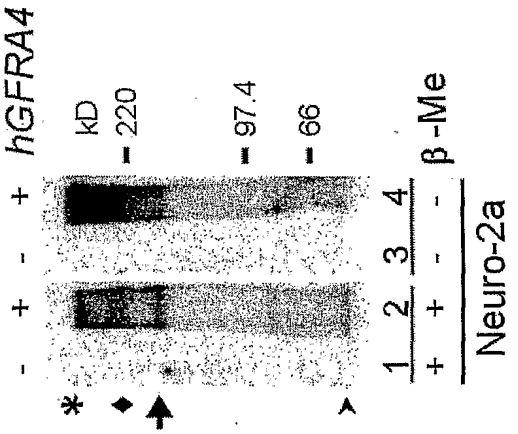

11A
Percentage of [125I] PSPN bound
100
80
60
40
20
0
GDNF
NRTN
ARTN
PSPN
0.1
1
10
100
1000
Cold ligand concentration (nM)
11B
PI-PLC
Cold PSPN
kD
220
97.4
66
46
30
21
1 2 3 4 5 6
hGFRA4
CHO

Fig. 11 A-B


11D
hGFRA4
kD
220
97.4
66
β-Me
1 2 3 4
+ + - -
Neuro-2a

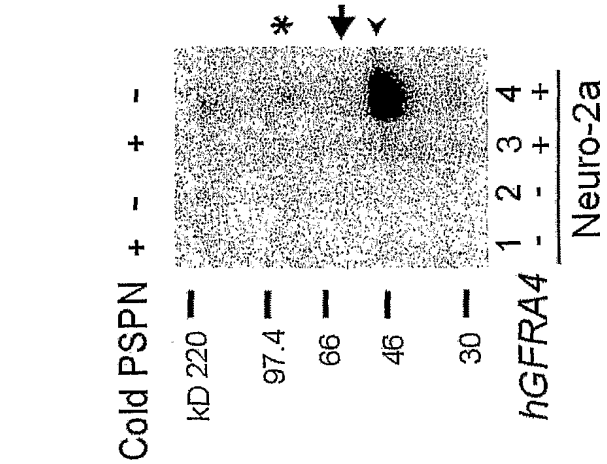
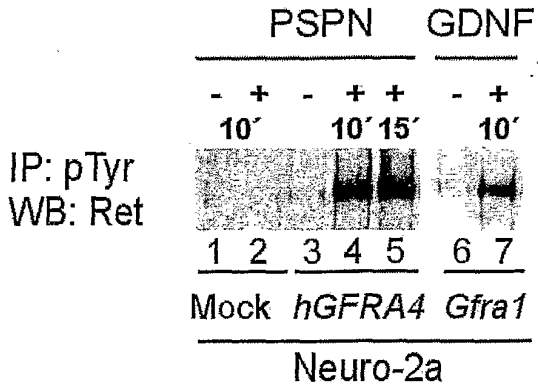

11C
Cold PSPN + - + -
kD 220
97.4
66
46
30
hGFRA4
1 2 3 4
- - + +
Neuro-2a

Fig. 11 C-D

11E



11F

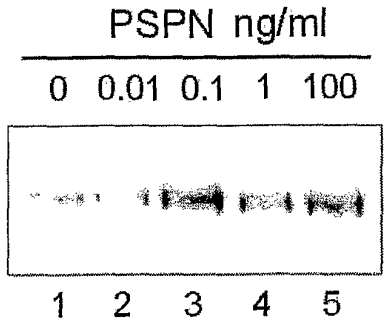


Fig. 11 E-F

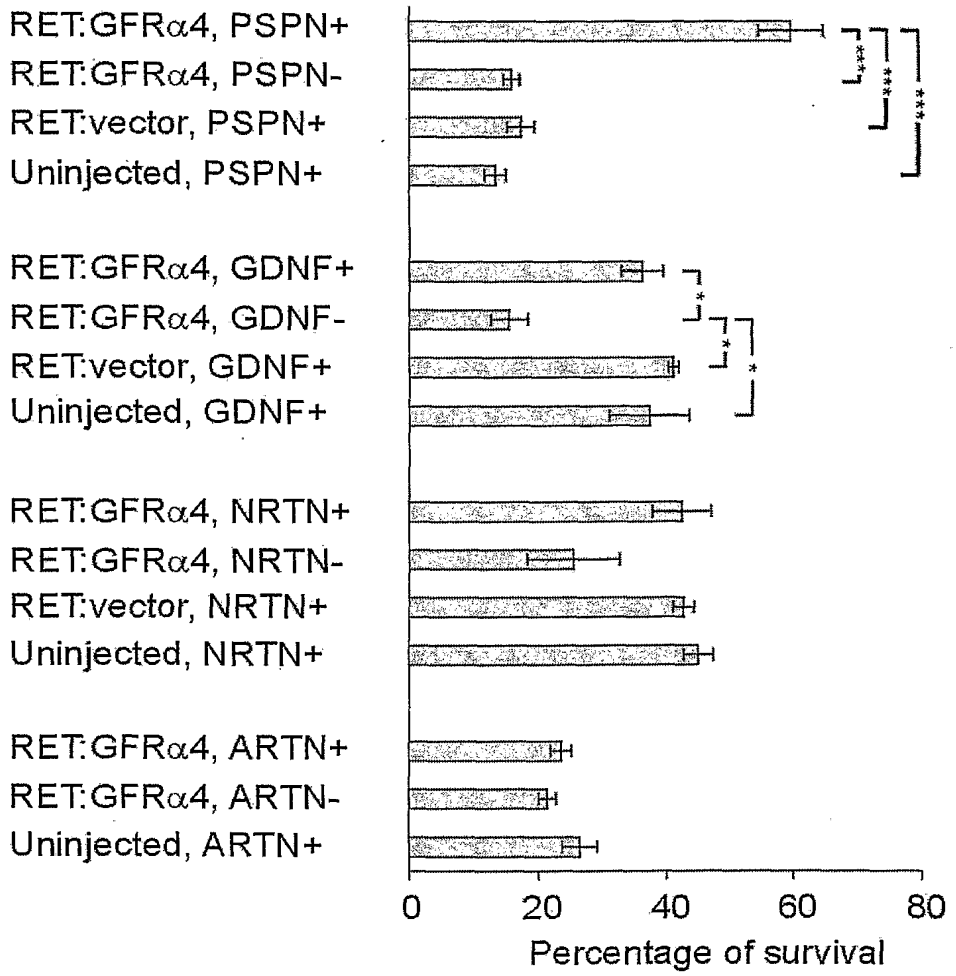

RET:GFRα4, PSPN+
RET:GFRα4, PSPN-
RET:vector, PSPN+
Uninjected, PSPN+
RET:GFRα4, GDNF+
RET:GFRα4, GDNF-
RET:vector, GDNF+
Uninjected, GDNF+
RET:GFRα4, NRTN+
RET:GFRα4, NRTN-
RET:vector, NRTN+
Uninjected, NRTN+
RET:GFRα4, ARTN+
RET:GFRα4, ARTN-
Uninjected, ARTN+
***
***
***
*
**
*
0
20
40
60
80
Percentage of survival

Fig. 12

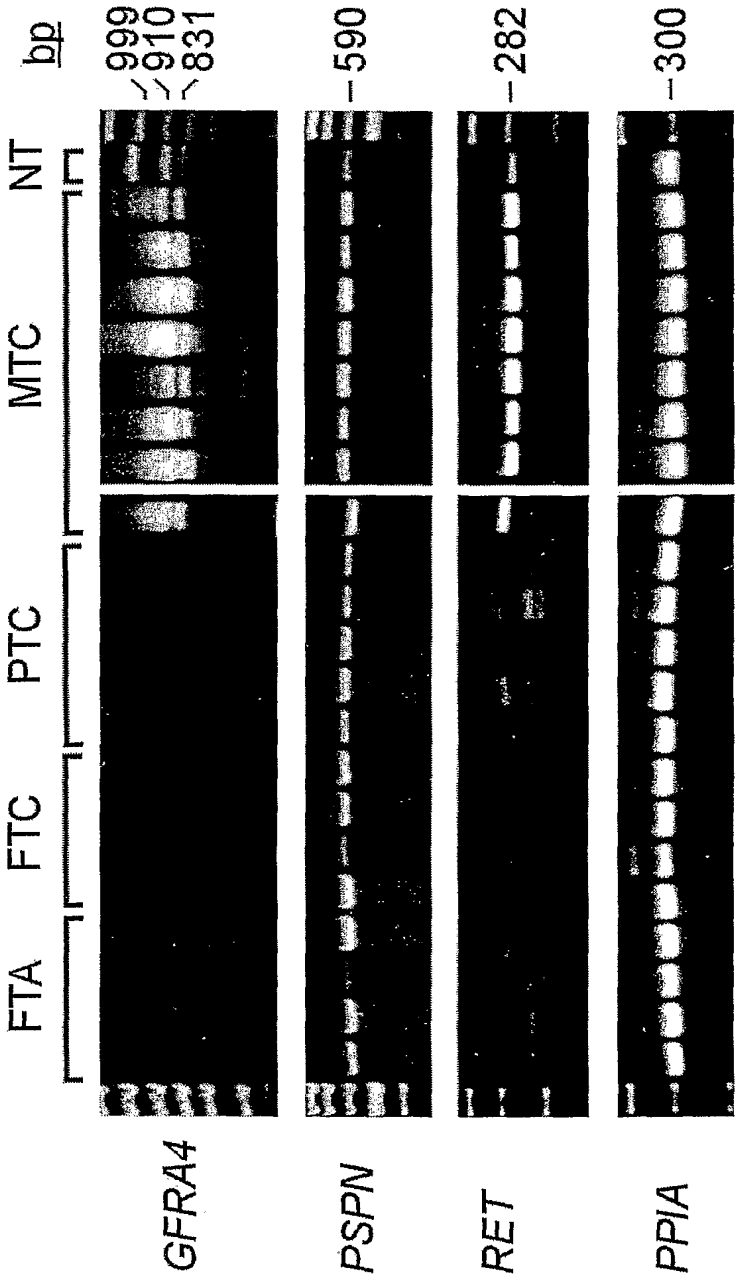

bp
999
910
831
590
282
300
FTA
FTC
PTC
MTC
NT
GFRA4
PSPN
RET
PPIA

Fig. 13

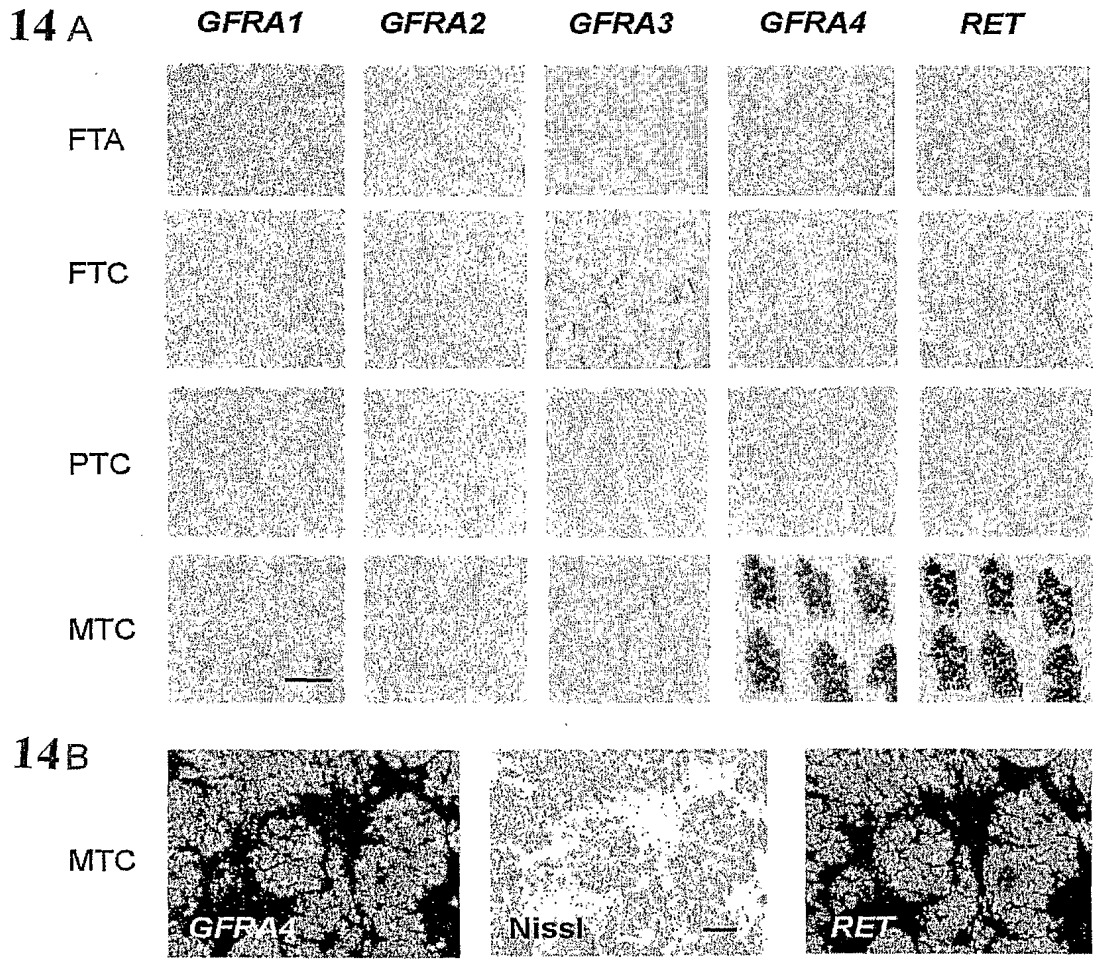
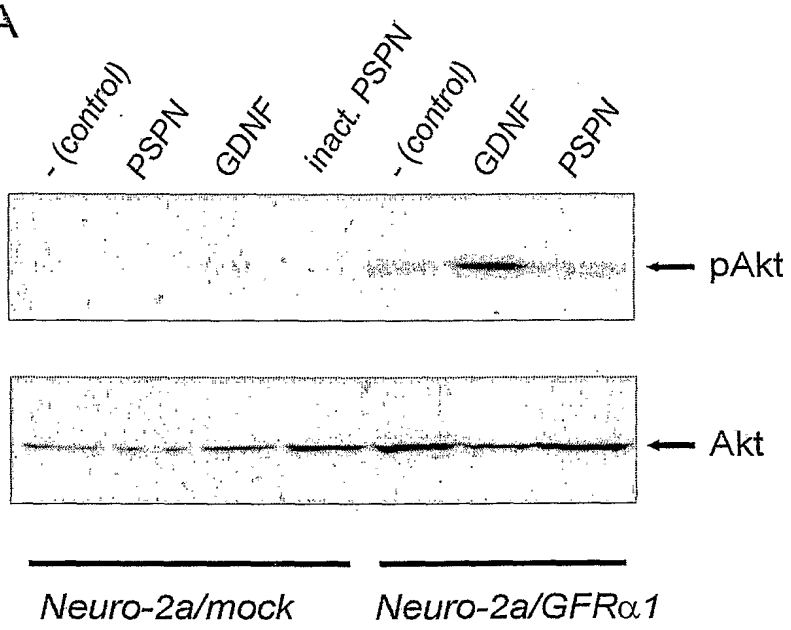

14 A
GFRA1
GFRA2
GFRA3
GFRA4
RET
FTA
FTC
PTC
MTC
14 B
MTC
GFRA4
Nissl
RET

Fig. 14

15A



15B

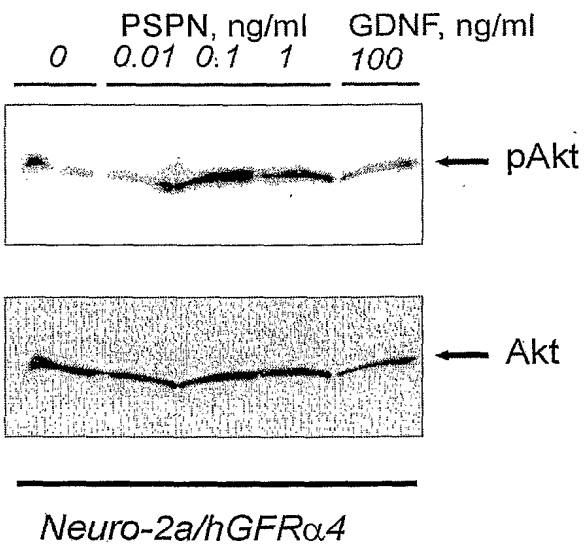
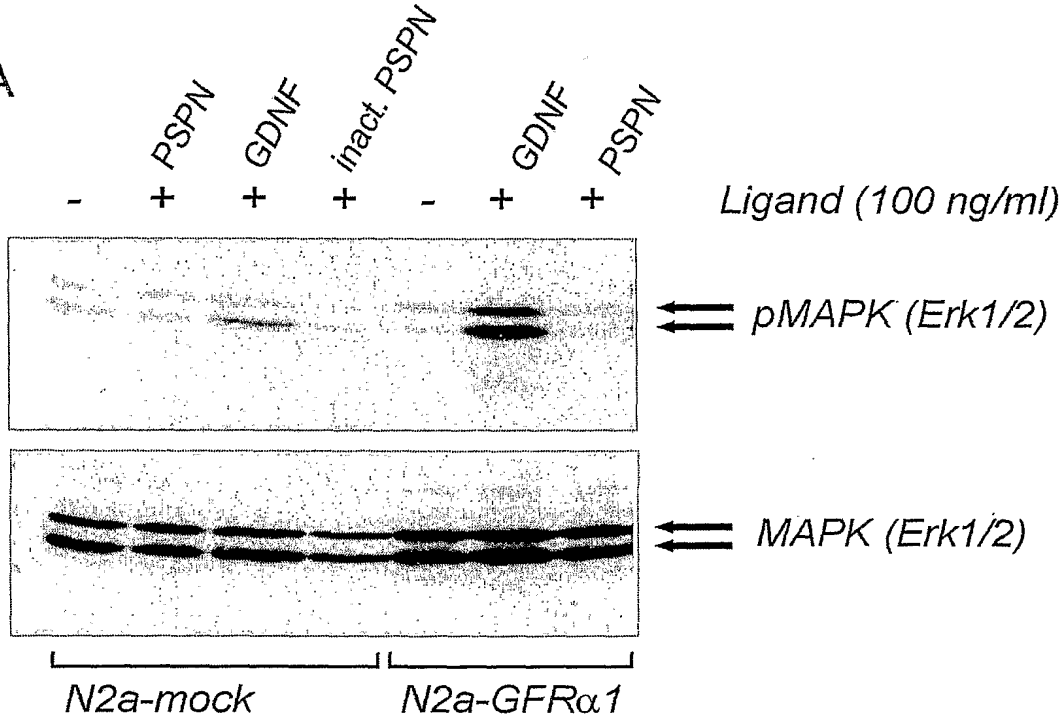


Fig. 15

16 A



16 B

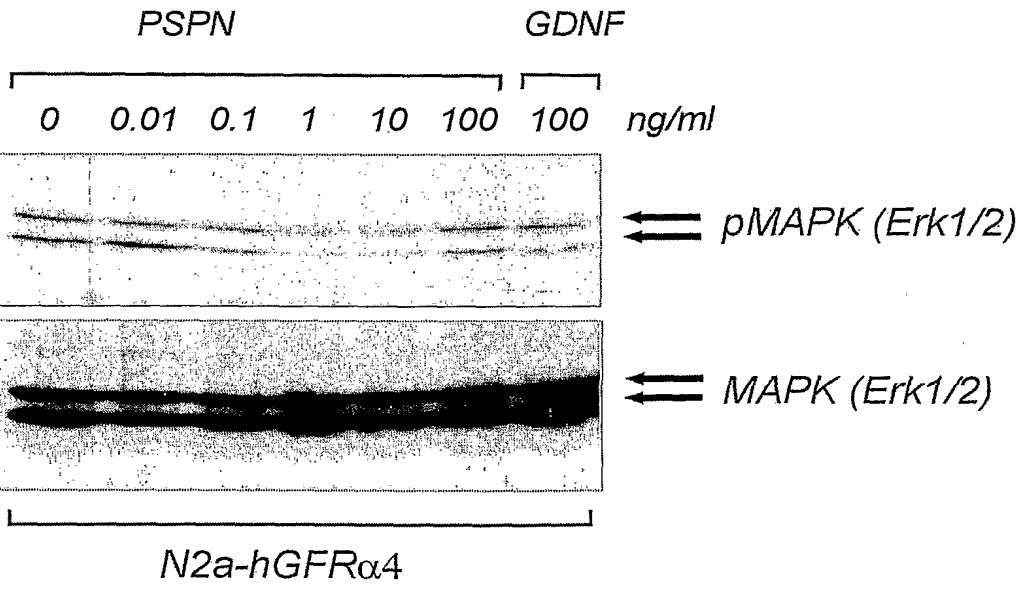
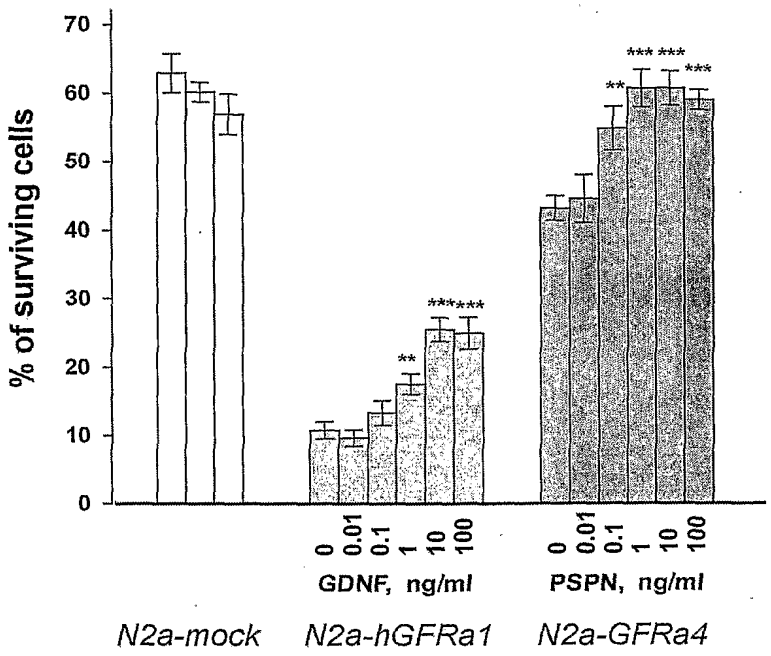


Fig. 16

17A



17B

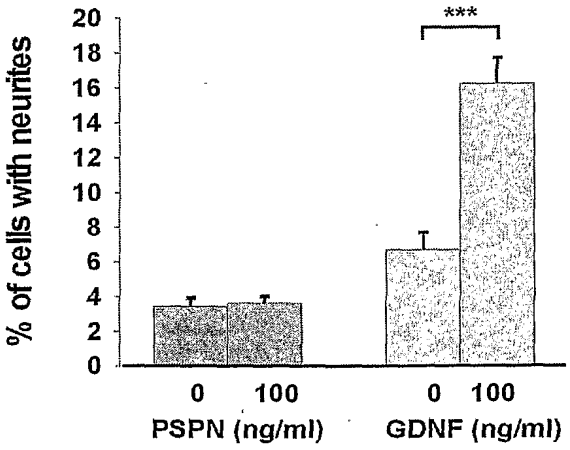


Fig. 17

Fig. 18A
Mouse Gfra4-[GPI]a1 cDNA sequence, length: 783

```
   1  atggcccact gcatggagtc tgcactgctg ctgttgttgc tgctggggtc
  51  tgcgagcttt accgacggga atcgctgcgt ggacgcggcc gaggcgtgta
 101  cagcagacga gcggtgccag cagctgcgct ctgagtacgt ggcacgatgc
 151  ctgggccggg cagcgcccgg gggcaggccg ggacccgggg gctgcgtgcg
 201  ctcccgctgc cgccgagccc tgcgccgctt cttcgcgcgt gggcctccgg
 251  cgctcacgca tgcgctgctc ttctgcggct gcgaaggctc cgcgtgcgcc
 301  gagcgccggc gccagacttt cgcgcccgcc tgcgcgttct ccggcccggg
 351  gttggtgccg ccctcttgcc tggagcccct ggagcgctgc gagcgcagcc
 401  gcctgtgccg gccccgtctc cttgccttcc aggcctcatg cgctcccgcg
 451  cccggctccc gcgaccgctg cccggaggag gggggcccgc gttgtctgcg
 501  cgtctacgca ggcctcatag gcaccgtggt caccccaac tacctggaca
 551  acgtgagcgc gcgcgttgcg ccctggtgcg gctgtgcggc cagtggaaac
 601  cggcgcgaag aatgcgaagc cttccgcaag ctctttacaa ggaacccctg
 651  cttggatggt gccatacaag cctttgacag cttgcagcca tcagttctgc
 701  aggaccagac tgctgggtgc tgtttcccgc gggtgtcctg gctgtatgca
 751  ctcactgccc tggctctcca ggccctgctc tga
```

Fig. 18B
Mouse GFRα4 GPI-linked isoform a1 amino-acid sequence, length: 261

```
   1  MAHCMESALL LLLLLGSASF TDGNRCVDAA EACTADERCQ QLRSEYVARC
  51  LGRAAPGGRP GPGGCVRSRC RRALRRFFAR GPPALTHALL FCGCEGSACA
 101  ERRRQTFAPA CAFSGPGLVP PSCLEPLERC ERSRLCRPRL LAFQASCAPA
 151  PGSRDRCPEE GGPRCLRVYA GLIGTVVTPN YLDNVSARVA PWCGCAASGN
 201  RREECEAFRK LFTRNPCLDG AIQAFDSLQP SVLQDQTAGC CFPRVSWLYA
 251  LTALALQALL*
```

Fig. 18

Fig. 19A
Mouse Gfra4 transcript a2 cDNA sequence, length:882

```
   1  atggcccact gcatggagtc tgcactgctg ctgttgttgc tgctggggtc
  51  tgcgagcttt accgacggga atcgctgcgt ggacgcggcc gaggcgtgta
 101  cagcagacga gcggtgccag cagctgcgct ctgagtacgt ggcacgatgc
 151  ctgggccggg cagcgcccgg gggcaggccg ggacccgggg gctgcgtgcg
 201  ctcccgctgc cgccgagccc tgcgccgctt cttcgcgcgt gggcctccgg
 251  cgctcacgca tgcgctgctc ttctgcggct gcgaaggctc cgcgtgcgcc
 301  gagcgccggc gccagacttt cgcgcccgcc tgcgcgttct ccggcccggg
 351  gttggtgccg ccctcttgcc tggagcccct ggagcgctgc gagcgcagcc
 401  gcctgtgccg gccccgtctc cttgccttcc aggcctcatg cgctcccgcg
 451  cccggctccc gcgaccgctg cccggaggag gggggcccgc gttgtctgcg
 501  cgtctacgca ggcctcatag gcaccgtggt caccccaac tacctggaca
 551  acgtgagcgc gcgcgttgcg ccctggtgcg gctgtgcggc cagtggaaac
 601  cggcgcgaag aatgcgaagc cttccgcaag ctctttacaa ggaacccctg
 651  cttggatggt gccatacaag cctttgacag cttgcagcca tcagttctgc
 701  aggaccagac tgctgggtgc tgtttcccgc gggcaaggca cgagtggcct
 751  gagaagagct ggaggcagaa acagtccttg ttttgtccta acgcccaagg
 801  tgtcctggct gtatgcactc actgccctgg ctctccaggc cctgctctga
 851  ttaggaacat gaaccgtgga cgacacagct ga
```

Fig. 19B
Mouse GFRα4 putative TM-isoform a2 amino-acid sequence, length:294

```
   1  MAHCMESALL LLLLLGSASF TDGNRCVDAA EACTADERCQ QLRSEYVARC
  51  LGRAAPGGRP GPGGCVRSRC RRALRRFFAR GPPALTHALL FCGCEGSACA
 101  ERRRQTFAPA CAFSGPGLVP PSCLEPLERC ERSRLCRPRL LAFQASCAPA
 151  PGSRDRCPEE GGPRCLRVYA GLIGTVVTPN YLDNVSARVA PWCGCAASGN
 201  RREECEAFRK LFTRNPCLDG AIQAFDSLQP SVLQDQTAGC CFPRARHEWP
 251  EKSWRQKQSL FCPNAQGVLA VCTHCPGSPG PALIRNMNRG RHS*
```

Fig. 19

Fig. 20A
Mouse Gfra4 transcript a3/a4 cDNA sequence, length:573

```
   1  ATGGCCCACT GCATGGAGTC TGCACTGCTG CTGTTGTTGC TGCTGGGGTC
  51  TGCGAGCTTT ACCGACGGGA ATCGCTGCGT GGACGCGGCC GAGGCGTGTA
 101  CAGCAGACGA GCGGTGCCAG CAGCTGCGCT CTGAGTACGT GGCACGATGC
 151  CTGGGCCGGG CAGCGCCCGG GGGCAGGCCG GGACCCGGGG GCTGCGTGCG
 201  CTCCCGCTGC CGCCGAGCCC TGCGCCGCTT CTTCGCGCGT GGGCCTCCGG
 251  CGCTCACGCA TGCGCTGCTC TTCTGCGGCT GCGAAGGCTC CGCGTGCGCC
 301  GAGCGCCGGC GCCAGACTTT CGCGCCCGCC TGCGCGTTCT CCGGCCCGGG
 351  GTTGGTGCCG CCCTCTTGCC TGGAGCCCCT GGAGCGCTGC GAGCGCAGCC
 401  GCCTGTGCCG gtgcgtgcgt gcggggcggg ctgggccgct cacccgcgtc
 451  cgggcgcgcg cagGCCCCGT CTCCTTGCCT TCCAGGCCTC ATGCGCTCCC
 501  GCGCCCGGCT CCCGCGACCG CTGCCCGGAG GAGGGGGGCC CGCGTTGTCT
 551  GCGCGTCTAC GCAGGCCTCA TAG
```

Fig. 20B
Mouse GFRα4 soluble isoform a3/4 amino-acid sequence, length: 191

```
   1  MAHCMESALL LLLLLGSASF TDGNRCVDAA EACTADERCQ QLRSEYVARC
  51  LGRAAPGGRP GPGGCVRSRC RRALRRFFAR GPPALTHALL FCGCEGSACA
 101  ERRRQTFAPA CAFSGPGLVP PSCLEPLERC ERSRLCRCVR AGRAGPLTRV
 151  RARAGPVSLP SRPHALPRPA PATAARRRGA RVVCASTQAS*
```

Fig. 20

Fig. 21A
Human *GFRA4a* cDNA sequence (GPI-linked splice form a), length: 810

```
   1  ATGGTCCGCT GCCTGGGGCC TGCGCTGCTG CTGCTGCTGT TACTGGGGTC
  51  GGCGAGCTCG GTCGGAGGGA ACCGATGTGT GGACGCGGCC GAAGCCTGCA
 101  CGGCGGACGC GCGGTGCCAG CGTTTGCGCT CCGAGTATGT GGCGCAGTGC
 151  CTGGGCCGGG CTGCGCAGGG GGGCTGTCCC CGCGCCCGCT GCCGCCGGGC
 201  CCTGCGCCGC TTCTTCGCCC GCGGGCCGCC CGCGCTCACC CACGCACTGC
 251  TCTTCTGCCC GTGCGCGGGC CCCGCGTGCG CCGAGCGTCG GCGCCAGACC
 301  TTCGTGCCCT CCTGCGCCTT TTCGGGGCCC GGCCCCGCGC CGCCCTCCTG
 351  CCTTGAGCCC TTAAACTTCT GCGAGCGCAG CCGGGTCTGC AGGCCTCGCC
 401  TCCTGGCCTT TCAGGTCTCG TGCACCCCAG CGCCCAGCGC CCCCGACGGC
 451  TGCCTGCTGG ACCAGGGCGC CCGCTGCCTG CGCGCCTACG CGGGCCTCGT
 501  GGGCACCGCC GTCACCCCTA ACTACGTGGA CAACGTGAGC GCGCGCGTGG
 551  CGCCCTGGTG CGACTGCGGA GCCAGCGGGA ACCGGCGTGA GGACTGCGAA
 601  GCCTTCCGGG GGCTCTTTAC CAGGAACCGC TGCTTGGATG GTGCCATTCA
 651  GGCCTTTGCC AGCGGGTGGC CCCCAGTCCT GCTGGACCAG CTGAACCCCC
 701  AGGGAGACCC GGAGCACAGC CTCCTGCAGG TGTCCTCCAC AGGCAGGGCC
 751  CTGGAGAGAC GCTCCCTGCT CTCCATACTT CCTGTCCTGG CTCTCCCGGC
 801  CCTGCTCTGA
```

Fig. 21B
Human GFRα4a amino-acid sequence (GPI-linked isoform a), length: 269

```
   1  MVRCLGPALL LLLLLGSASS VGGNRCVDAA EACTADARCQ RLRSEYVAQC
  51  LGRAAQGGCP RARCRRALRR FFARGPPALT HALLFCPCAG PACAERRRQT
 101  FVPSCAFSGP GPAPPSCLEP LNFCERSRVC RPRLLAFQVS CTPAPSAPDG
 151  CLLDQGARCL RAYAGLVGTA VTPNYVDNVS ARVAPWCDCG ASGNRREDCE
 201  AFRGLFTRNR CLDGAIQAFA SGWPPVLLDQ LNPQGDPEHS LLQVSSTGRA
 251  LERRSLLSIL PVLALPALL*
```

Fig. 21

Fig. 22A
Human GFRA4b splice form cDNA sequence, length: 900

ATGGTCCGCTGCCTGGGGCCTGCGCTGCTGCTGCTGCTGTTACTGGGGTCGGCGAGCTCGGTCGGAGGG
AACCGATGTGTGGACGCGGCCGAAGCCTGCACGGCGGACGCGCGGTGCCAGCGTTTGCGCTCCGAGTAT
GTGGCGCAGTGCCTGGGCCGGGCTGCGCAGGGGGGCTGTCCCCGCGCCCGCTGCCGCCGGGCCCTGCGC
CGCTTCTTCGCCCGCGGGCCGCCCGCGCTCACCCACGCACTGCTCTTCTGCCCGTGCGCGGGCCCCGCG
TGCGCCGAGCGTCGGCGCCAGACCTTCGTGCCCTCCTGCGCCTTTTCGGGGCCCGGCCCCGCGCCGCCC
TCCTGCCTTGAGCCCTTAAACTTCTGCGAGCGCAGCCGGGTCTGCAGGTGCGCGCGGGCGGCGGCGGGG
CCGTGGCGAGGGTGGGGACGGGGCCTCTCTCCGGCTCACCGCCCTCCCGCCGCGCAGGCCTCGCCTCCT
GGCCTTTCAGGTCTCGTGCACCCCAGCGCCCAGCGCCCCCGACGGCTGCCTGCTGGACCAGGGCGCCCG
CTGCCTGCGCGCCTACGCGGGCCTCGTGGggtccccgcaggcaccgccgtcacccctaactacgtggac
aacgtgagcgcgcgcgtggcgccctggtgcgactgcggagccagcgggaaccggcgtgaggactgcgaa
gccttccgggggctctttaccaggaaccgctgcttggATGGTGCCATTCAGGCCTTTGCCAGCGGGTGG
CCCCCAGTCCTGCTGGACCAGCTGAACCCCCAGGGAGACCCGGAGCACAGCCTCCTGCAGGTGTCCTCC
ACAGGCAGGGCCCTGGAGAGACGCTCCCTGCTCTCCATACTTCCTGTCCTGGCTCTCCCGGCCCTGCTC
TGA

Fig. 22B
Human GFRα4b (putative GPI-anchored isoform b) amino-acid sequence, length:299

The non-homologous part (from GFRα4a) is indicated in grey, this corresponds to the translated intron 2, exon 3 (in other frame), and four amino acids (GVPA) from intron 3.

MVRCLGPALLLLLLLGSASSVGGNRCVDAAEACTADARCQRLRSEYVAQCLGRAAQGGCPRARCRRALR
RFFARGPPALTHALLFCPCAGPACAERRRQTFVPSCAFSGPGPAPPSCLEPLNFCERSRVCRCARAAAG
PWRGWGRGLSPAHRPPAAQASPPGLSGLVHPSAQRPRRLPAGPGRPLPARLRGPRGVPAGTAVTPNYVD
NVSARVAPWCDCGASGNRREDCEAFRGLFTRNRCLDGAIQAFASGWPPVLLDQLNPQGDPEHSLLQVSS
TGRALERRSLLSILPVLALPALL*

Fig. 22

Fig. 23A
Human *GFRA4c* (putative soluble form) cDNA sequence, length: 549

```
   1  ATGGTCCGCT GCCTGGGGCC TGCGCTGCTG CTGCTGCTGT TACTGGGGTC
  51  GGCGAGCTCG GTCGGAGGGA ACCGATGTGT GGACGCGGCC GAAGCCTGCA
 101  CGGCGGACGC GCGGTGCCAG CGTTTGCGCT CCGAGTATGT GGCGCAGTGC
 151  CTGGGCCGGG CTGCGCAGGG GGGCTGTCCC CGCGCCCGCT GCCGCCGGGC
 201  CCTGCGCCGC TTCTTCGCCC GCGGGCCGCC CGCGCTCACC CACGCACTGC
 251  TCTTCTGCCC GTGCGCGGGC CCCGCGTGCG CCGAGCGTCG GCGCCAGACC
 301  TTCGTGCCCT CCTGCGCCTT TTCGGGGCCC GGCCCCGCGC CGCCCTCCTG
 351  CCTTGAGCCC TTAAACTTCT GCGAGCGCAG CCGGGTCTGC AGGCCTCGCC
 401  TCCTGGCCTT TCAGGTCTCG TGCACCCCAG CGCCCAGCGC CCCCGACGGC
 451  TGCCTGCTGG ACCAGGGCGC CCGCTGCCTG CGCGCCTACG CGGGCCTCGT
 501  GGGGTCCCCG CAGGCACCGC CGTCACCCCT AACTACGTGG ACAACGTGA
```

Fig. 23B
Human GFRα4c (putative soluble isoform c) amino-acid sequence, length: 182

```
   1  MVRCLGPALL LLLLLGSASS VGGNRCVDAA EACTADARCQ RLRSEYVAQC
  51  LGRAAQGGCP RARCRRALRR FFARGPPALT HALLFCPCAG PACAERRRQT
 101  FVPSCAFSGP GPAPPSCLEP LNFCERSRVC RPRLLAFQVS CTPAPSAPDG
 151  CLLDQGARCL RAYAGLVGSP QAPPSPLTTW TT*
```

Fig. 23

Human *GFRA4* locus genomic sequence

..CTATCAGACTAGGGCTCTGCCAGCCATCCTTCTCTGTTGAAGGTCCAGC**ATG**GTCCGCTGCCTGG
GGCCTGCGCTGCTGCTGCTGCTGTTACTGG**gt**..longintron..**ag**GGTCGGCGAGCTCG
GTCGGAGGGAACCGATGTGTGGACGCGGCCGAAGCCTGCACGGCGGACGCGCGGTGCCAGCG
TTTGCGCTCCGAGTATGTGGCGCAGTGCCTGGGCCGGGCTGCGCAGGGGGGCTGTCCCCGCG
CCCGCTGCCGCCGGGCCCTGCGCCGCTTCTTCGCCCGCGGGCCGCCCGCGCTCACCCACGCA
CTGCTCTTCTGCCCGTGCGCGGGCCCCGCGTGCGCCGAGCGTCGGCGCCAGACCTTCGTGCC
CTCCTGCGCCTTTTCGGGGCCCGGCCCCGCGCCGCCCTCCTGCCTTGAGCCCTTAAACTTCT
GCGAGCGCAGCCGGGTCTGCAG**gtgcgcgcgggcggcggcggggccgtggcgagggtgggga**
cggggcctctctccggctcaccgccctcccgccgcgcagGCCTCGCCTCCTGGCCTTTCAGG
TCTCGTGCACCCCAGCGCCCAGCGCCCCCGACGGCTGCCTGCTGGACCAGGGCGCCCGCTGC
CTGCGCGCCTACGCGGGCCTCGTGG**gtacgcgcggccgggatccgggcgagggcgggggttc**
tccaggggatatctccgcccgggtgggccgatgacttcgccctcag*ggtccccgcag*GCACC
GCCGTCACCCCTAACTACGTGGACAACGTGAGCGCGCGCGTGGCGCCCTGGTGCGACTGCGG
AGCCAGCGGGAACCGGCGTGAGGACTGCGAAGCCTTCCGGGGGCTCTTTACCAGGAACCGCT
GCTTGG**gtgaggggcccgggggggagtggaggggagtgggggcggcgcttactgccccctc**
ccaagccgcctggctgggagccattttagaggggagaatggaagactgtacagttgagtcac
tctgtcacagctgtgcttattgttttgttattcctcaccacacaccttctgtccaaggagcc
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cagctaagtccaccctggacccctccctccatagATGGTGCCATTCAGGCCTTTGCCAGCGG
GTGGCCCCCAGTCCTGCTGGACCAGCTGAACCCCCAGGGAGACCCGGAGCACAGCCTCCTGC
AG**gtaggtgcagggaggggagggtgagctggcacctcccccactgtcaccttcacacccttc**
cgtccctggtgggcctgggtggaggcatgaagggcctggggtgggggtgcaggcagagggca
gagacaggcttttgcctcaagtctgcacttggctcccaccccccaagGTGTCCTCCACAGGCA
GGGCCCTGGAGAGACGCTCCCTGCTCTCCATACTTCCTGTCCTGGCTCTCCCGGCCCTGCTC
TGATTAGGACAGCGACCTCGGATAGCACAGCCAGCTACTCCACCCTGCCTGCCTGGGCCGCC
TCTGTGGCCTACTGGCCCCTTGAGAAGGGACTGGCTTACCCCCCAAGCCGGCCCTGGTGCTT
TCACTCCGCTGCCCTTTGTAGGTTTGGACACCCTGTGTGCCGTCCCCTGGGGCAAGGGATGT
AGGCTGGGGCCTGACTGTAAAGCCCCCGTCTCCCTGTCAGGAGGCATCTTGGTTGTAAGTCC
CTTTATTCACAGACCTTGAGACCACTGGGGTCTCCCACAAGGTGGGGTCAGGAGAGGTCACT
TTTGTAGCTGAGACCTCTCTGGAGACCCAGATCCCCTAGAGCAGGTCAGAGACATCCAGAAT
CCCAGAATTCTAGGAAATTGTATCAGCCTCCCAAGCATATAACCCCCTAAGGAATCCATCGG
ACAAGACCCGTCTAACACTGCATCCTCCCAACTGGGGCATTACCCACCATTGTAGCCACCTG
TGCACCATGACATGCTGGGCAGAGTCTTTCCTGTTCCCCATATGCTGCCTGTGGAGGAAAAC
CTNGCAAGGGGCNA..

Fig. 24

SEQUENCE LISTING

<110> AIRAKSINEN, MATTI
SAARMA, MART

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<130> 9M34PC

<140>
<141>

<150> FI 20000394
<151> 2000-02-21

<160> 50

<170> PatentIn Ver. 2.1

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<220>
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<400> 1

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Ser Ala Ser Phe Thr Asp Gly Asn Arg Cys Val Asp Ala Ala Glu Ala
             20                  25                  30

Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu Tyr Val Ala
         35                  40                  45

Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly Pro Gly Gly
     50                  55                  60

Cys Val Arg Ser Arg Cys Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg
 65                  70                  75                  80

Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly Cys Glu Gly
                 85                  90                  95

Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro Ala Cys Ala
```

```
            100                 105                 110

Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu Pro Leu Glu
        115                 120                 125

Arg Cys Glu Arg Ser Arg Leu Cys Arg Pro Arg Leu Leu Ala Phe Gln
    130                 135                 140

Ala Ser Cys Ala Pro Ala Pro Gly Ser Arg Asp Arg Cys Pro Glu Glu
145                 150                 155                 160

Gly Gly Pro Arg Cys Leu Arg Val Tyr Ala Gly Leu Ile Gly Thr Val
                165                 170                 175

Val Thr Pro Asn Tyr Leu Asp Asn Val Ser Ala Arg Val Ala Pro Trp
            180                 185                 190

Cys Gly Cys Ala Ala Ser Gly Asn Arg Arg Glu Glu Cys Glu Ala Phe
        195                 200                 205

Arg Lys Leu Phe Thr Arg Asn Pro Cys Leu Asp Gly Ala Ile Gln Ala
    210                 215                 220

Phe Asp Ser Leu Gln Pro Ser Val Leu Gln Asp Gln Thr Ala Gly Cys
225                 230                 235                 240

Cys Phe Pro Arg Val Ser Trp Leu Tyr Ala Leu Thr Ala Leu Ala Leu
                245                 250                 255

Gln Ala Leu Leu
            260
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<210> 2
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<212> PRT
<213> Mus musculus

<220>
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<400> 2

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Ser Ala Ser Phe Thr Asp Gly Asn Arg Cys Val Asp Ala Ala Glu Ala
             20                  25                  30
```

```
Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu Tyr Val Ala
         35                  40                  45

Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly Pro Gly Gly
     50                  55                  60

Cys Val Arg Ser Arg Cys Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg
 65                  70                  75                  80

Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly Cys Glu Gly
                 85                  90                  95

Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro Ala Cys Ala
            100                 105                 110

Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu Pro Leu Glu
        115                 120                 125

Arg Cys Glu Arg Ser Arg Leu Cys Arg Pro Arg Leu Leu Ala Phe Gln
    130                 135                 140

Ala Ser Cys Ala Pro Ala Pro Gly Ser Arg Asp Arg Cys Pro Glu Glu
145                 150                 155                 160

Gly Gly Pro Arg Cys Leu Arg Val Tyr Ala Gly Leu Ile Gly Thr Val
                165                 170                 175

Val Thr Pro Asn Tyr Leu Asp Asn Val Ser Ala Arg Val Ala Pro Trp
            180                 185                 190

Cys Gly Cys Ala Ala Ser Gly Asn Arg Arg Glu Glu Cys Glu Ala Phe
        195                 200                 205

Arg Lys Leu Phe Thr Arg Asn Pro Cys Leu Asp Gly Ala Ile Gln Ala
    210                 215                 220

Phe Asp Ser Leu Gln Pro Ser Val Leu Gln Asp Gln Thr Ala Gly Cys
225                 230                 235                 240

Cys Phe Pro Arg Ala Arg His Glu Trp Pro Glu Lys Ser Trp Arg Gln
                245                 250                 255

Lys Gln Ser Leu Phe Cys Pro Asn Ala Gln Gly Val Leu Ala Val Cys
            260                 265                 270

Thr His Cys Pro Gly Ser Pro Gly Pro Ala Leu Ile Arg Asn Met Asn
        275                 280                 285
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Arg Gly Arg His Ser
290

<210> 3
<211> 190
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<213> Mus musculus

<220>
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20 25 30

Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu Tyr Val Ala
35 40 45

Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly Pro Gly Gly
50 55 60

Cys Val Arg Ser Arg Cys Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg
65 70 75 80

Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly Cys Glu Gly
85 90 95

Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro Ala Cys Ala
100 105 110

Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu Pro Leu Glu
115 120 125

Arg Cys Glu Arg Ser Arg Leu Cys Arg Val Cys Arg Ala Gly Arg Ala
130 135 140

Gly Pro Leu Thr Arg Val Arg Ala Arg Ala Gly Pro Val Ser Leu Pro
145 150 155 160

Ser Arg Pro His Ala Leu Pro Arg Pro Ala Pro Ala Thr Ala Ala Arg
165 170 175

Arg Arg Gly Ala Arg Val Val Cys Ala Ser Thr Gln Ala Ser
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<212> PRT
<213> Homo sapiens

<220>
<223> GPI-anchored isoform a

<400> 4

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Ser Ala Ser Ser Val Gly Gly Asn Arg Cys Val Asp Ala Ala Glu Ala
             20                  25                  30

Cys Thr Ala Asp Ala Arg Cys Gln Arg Leu Arg Ser Glu Tyr Val Ala
         35                  40                  45

Gln Cys Leu Gly Arg Ala Ala Gln Gly Gly Cys Pro Arg Ala Arg Cys
     50                  55                  60

Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg Gly Pro Pro Ala Leu Thr
 65                  70                  75                  80

His Ala Leu Leu Phe Cys Pro Cys Ala Gly Pro Ala Cys Ala Glu Arg
                 85                  90                  95

Arg Arg Gln Thr Phe Val Pro Ser Cys Ala Phe Ser Gly Pro Gly Pro
            100                 105                 110

Ala Pro Pro Ser Cys Leu Glu Pro Leu Asn Phe Cys Glu Arg Ser Arg
        115                 120                 125

Val Cys Arg Pro Arg Leu Leu Ala Phe Gln Val Ser Cys Thr Pro Ala
    130                 135                 140

Pro Ser Ala Pro Asp Gly Cys Leu Leu Asp Gln Gly Ala Arg Cys Leu
145                 150                 155                 160

Arg Ala Tyr Ala Gly Leu Val Gly Thr Ala Val Thr Pro Asn Tyr Val
                165                 170                 175

Asp Asn Val Ser Ala Arg Val Ala Pro Trp Cys Asp Cys Gly Ala Ser
            180                 185                 190

Gly Asn Arg Arg Glu Asp Cys Glu Ala Phe Arg Gly Leu Phe Thr Arg
```

```
        195                 200                 205

Asn Arg Cys Leu Asp Gly Ala Ile Gln Ala Phe Ala Ser Gly Trp Pro
    210                 215                 220

Pro Val Leu Leu Asp Gln Leu Asn Pro Gln Gly Asp Pro Glu His Ser
225                 230                 235                 240

Leu Leu Gln Val Ser Ser Thr Gly Arg Ala Leu Glu Arg Arg Ser Leu
                245                 250                 255

Leu Ser Ile Leu Pro Val Leu Ala Leu Pro Ala Leu Leu
            260                 265
```

<210> 5
<211> 299
<212> PRT
<213> Homo sapiens

<220>
<223> Putative GPI-anchored isoform b

<400> 5

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Met Val Arg Cys Leu Gly Pro Ala Leu Leu Leu Leu Leu Leu Leu Gly
  1               5                  10                  15

Ser Ala Ser Ser Val Gly Gly Asn Arg Cys Val Asp Ala Ala Glu Ala
             20                  25                  30

Cys Thr Ala Asp Ala Arg Cys Gln Arg Leu Arg Ser Glu Tyr Val Ala
         35                  40                  45

Gln Cys Leu Gly Arg Ala Ala Gln Gly Gly Cys Pro Arg Ala Arg Cys
     50                  55                  60

Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg Gly Pro Pro Ala Leu Thr
 65                  70                  75                  80

His Ala Leu Leu Phe Cys Pro Cys Ala Gly Pro Ala Cys Ala Glu Arg
                85                  90                  95

Arg Arg Gln Thr Phe Val Pro Ser Cys Ala Phe Ser Gly Pro Gly Pro
            100                 105                 110

Ala Pro Pro Ser Cys Leu Glu Pro Leu Asn Phe Cys Glu Arg Ser Arg
        115                 120                 125
```

```
Val Cys Arg Cys Ala Arg Ala Ala Ala Gly Pro Trp Arg Gly Trp Gly
    130                 135                 140

Arg Gly Leu Ser Pro Ala His Arg Pro Pro Ala Ala Gln Ala Ser Pro
145                 150                 155                 160

Pro Gly Leu Ser Gly Leu Val His Pro Ser Ala Gln Arg Pro Arg Arg
                165                 170                 175

Leu Pro Ala Gly Pro Gly Arg Pro Leu Pro Ala Arg Leu Arg Gly Pro
            180                 185                 190

Arg Gly Val Pro Ala Gly Thr Ala Val Thr Pro Asn Tyr Val Asp Asn
        195                 200                 205

Val Ser Ala Arg Val Ala Pro Trp Cys Asp Cys Gly Ala Ser Gly Asn
    210                 215                 220

Arg Arg Glu Asp Cys Glu Ala Phe Arg Gly Leu Phe Thr Arg Asn Arg
225                 230                 235                 240

Cys Leu Asp Gly Ala Ile Gln Ala Phe Ala Ser Gly Trp Pro Pro Val
                245                 250                 255

Leu Leu Asp Gln Leu Asn Pro Gln Gly Asp Pro Glu His Ser Leu Leu
            260                 265                 270

Gln Val Ser Ser Thr Gly Arg Ala Leu Glu Arg Arg Ser Leu Leu Ser
        275                 280                 285

Ile Leu Pro Val Leu Ala Leu Pro Ala Leu Leu
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```

<210> 6
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Met Val Arg Cys Leu Gly Pro Ala Leu Leu Leu Leu Leu Leu Leu Gly
  1               5                  10                  15

Ser Ala Ser Ser Val Gly Gly Asn Arg Cys Val Asp Ala Ala Glu Ala
            20                  25                  30
```

```
Cys Thr Ala Asp Ala Arg Cys Gln Arg Leu Arg Ser Glu Tyr Val Ala
         35                  40                  45

Gln Cys Leu Gly Arg Ala Ala Gln Gly Gly Cys Pro Arg Ala Arg Cys
     50                  55                  60

Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg Gly Pro Pro Ala Leu Thr
 65                  70                  75                  80

His Ala Leu Leu Phe Cys Pro Cys Ala Gly Pro Ala Cys Ala Glu Arg
                 85                  90                  95

Arg Arg Gln Thr Phe Val Pro Ser Cys Ala Phe Ser Gly Pro Gly Pro
            100                 105                 110

Ala Pro Pro Ser Cys Leu Glu Pro Leu Asn Phe Cys Glu Arg Ser Arg
        115                 120                 125

Val Cys Arg Pro Arg Leu Leu Ala Phe Gln Val Ser Cys Thr Pro Ala
    130                 135                 140

Pro Ser Ala Pro Asp Gly Cys Leu Leu Asp Gln Gly Ala Arg Cys Leu
145                 150                 155                 160

Arg Ala Tyr Ala Gly Leu Val Gly Ser Pro Gln Ala Pro Pro Ser Pro
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Leu Thr Thr Trp Thr Thr
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<210> 7
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<212> DNA
<213> Mus musculus

<220>
<223> Splice form (a1) for GPI linked protein

<400> 7

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accgacggga atcgctgcgt ggacgcggcc gaggcgtgta cagcagacga gcggtgccag 120
cagctgcgct ctgagtacgt ggcacgatgc ctgggccggg cagcgcccgg gggcaggccg 180
ggacccgggg gctgcgtgcg ctcccgctgc cgccgagccc tgcgccgctt cttcgcgcgt 240
gggcctccgg cgctcacgca tgcgctgctc ttctgcggct gcgaaggctc cgcgtgcgcc 300
gagcgccggc gccagacttt cgcgcccgcc tgcgcgttct ccggcccggg gttggtgccg 360
ccctcttgcc tggagcccct ggagcgctgc gagcgcagcc gcctgtgccg gccccgtctc 420
```

cttgccttcc aggcctcatg cgctcccgcg cccggctccc gcgaccgctg cccggaggag 480
gggggcccgc gttgtctgcg cgtctacgca ggcctcatag gcaccgtggt cacccccaac 540
tacctggaca acgtgagcgc gcgcgttgcg ccctggtgcg gctgtgcggc cagtggaaac 600
cggcgcgaag aatgcgaagc cttccgcaag ctctttacaa ggaacccctg cttggatggt 660
gccatacaag cctttgacag cttgcagcca tcagttctgc aggaccagac tgctgggtgc 720
tgtttcccgc gggtgtcctg gctgtatgca ctcactgccc tggctctcca ggccctgctc 780
tga 783

<210> 8
<211> 882
<212> DNA
<213> Mus musculus

<220>
<223> Splice form (a2) (for putative transmembrane anchored protein)

<400> 8
atggcccact gcatggagtc tgcactgctg ctgttgttgc tgctggggtc tgcgagcttt 60
accgacggga atcgctgcgt ggacgcggcc gaggcgtgta cagcagacga gcggtgccag 120
cagctgcgct ctgagtacgt ggcacgatgc ctgggccggg cagcgcccgg gggcaggccg 180
ggacccgggg gctgcgtgcg ctcccgctgc cgccgagccc tgcgccgctt cttcgcgcgt 240
gggcctccgg cgctcacgca tgcgctgctc ttctgcggct gcgaaggctc cgcgtgcgcc 300
gagcgccggc gccagacttt cgcgcccgcc tgcgcgttct ccggcccggg gttggtgccg 360
ccctcttgcc tggagccccc ggagcgctgc gagcgcagcc gcctgtgccg gccccgtctc 420
cttgccttcc aggcctcatg cgctcccgcg cccggctccc gcgaccgctg cccggaggag 480
gggggcccgc gttgtctgcg cgtctacgca ggcctcatag gcaccgtggt cacccccaac 540
tacctggaca acgtgagcgc gcgcgttgcg ccctggtgcg gctgtgcggc cagtggaaac 600
cggcgcgaag aatgcgaagc cttccgcaag ctctttacaa ggaacccctg cttggatggt 660
gccatacaag cctttgacag cttgcagcca tcagttctgc aggaccagac tgctgggtgc 720
tgtttcccgc gggcaaggca cgagtggcct gagaagagct ggaggcagaa acagtccttg 780
ttttgtccta acgcccaagg tgtcctggct gtatgcactc actgccctgg ctctccaggc 840
cctgctctga ttaggaacat gaaccgtgga cgacacagct ga 882

<210> 9
<211> 573
<212> DNA
<213> Mus musculus

<220>
<223> Splice form (a3/a4) (for secreted protein)

<400> 9
atggcccact gcatggagtc tgcactgctg ctgttgttgc tgctggggtc tgcgagcttt 60
accgacggga atcgctgcgt ggacgcggcc gaggcgtgta cagcagacga gcggtgccag 120
cagctgcgct ctgagtacgt ggcacgatgc ctgggccggg cagcgcccgg gggcaggccg 180
ggacccgggg gctgcgtgcg ctcccgctgc cgccgagccc tgcgccgctt cttcgcgcgt 240
gggcctccgg ctcgcacgca tgcgctgctc ttctgcggct gcgaaggctc cgcgtgcgcc 300

gagcgccggc gccagacttt cgcgcccgcc tgcgcgttct ccggcccggg gttggtgccg 360
ccctcttgcc tggagcccct ggagcgctgc gagcgcagcc gcctgtgccg gtgcgtgcgt 420
gcggggcggg ctgggccgct cacccgcgtc cgggcgcgcg caggccccgt ctccttgcct 480
tccaggcctc atgcgctccc gcgcccggct cccgcgaccg ctgcccggag gaggggggcc 540
cgcgttgtct gcgcgtctac gcaggcctca tag 573

<210> 10
<211> 810
<212> DNA
<213> Homo sapiens

<220>
<223> Coding sequence of the splice form a for
GPI-anchored protein

<400> 10
atggtccgct gcctggggcc tgcgctgctg ctgctgctgt tactggggtc ggcgagctcg 60
gtcggaggga accgatgtgt ggacgcggcc gaagcctgca cggcggacgc gcggtgccag 120
cgtttgcgct ccgagtatgt ggcgcagtgc ctgggccggg ctgcgcaggg gggctgtccc 180
cgcgcccgct gccgccgggc cctgcgccgc ttcttcgccc gcgggccgcc cgcgctcacc 240
cacgcactgc tcttctgccc gtgcgcgggc cccgcgtgcg ccgagcgtcg gcgccagacc 300
ttcgtgccct cctgcgcctt ttcggggccc ggccccgcgc cgccctcctg ccttgagccc 360
ttaaacttct gcgagcgcag ccgggtctgc aggcctcgcc tcctggcctt tcaggtctcg 420
tgcaccccag cgcccagcgc ccccgacggc tgcctgctgg accagggcgc ccgctgcctg 480
cgcgcctacg cgggcctcgt gggcaccgcc gtcaccccta actacgtgga caacgtgagc 540
gcgcgcgtgg cgccctggtg cgactgcgga gccagcggga accggcgtga ggactgcgaa 600
gccttccggg ggctctttac caggaaccgc tgcttggatg gtgccattca ggcctttgcc 660
agcgggtggc ccccagtcct gctggaccag ctgaaccccc agggagaccc ggagcacagc 720
ctcctgcagg tgtcctccac aggcagggcc ctggagagac gctccctgct ctccatactt 780
cctgtcctgg ctctcccggc cctgctctga 810

<210> 11
<211> 900
<212> DNA
<213> Homo sapiens

<220>
<223> Splice form b

<400> 11
atggtccgct gcctggggcc tgcgctgctg ctgctgctgt tactggggtc ggcgagctcg 60
gtcggaggga accgatgtgt ggacgcggcc gaagcctgca cggcggacgc gcggtgccag 120
cgtttgcgct ccgagtatgt ggcgcagtgc ctgggccggg ctgcgcaggg gggctgtccc 180
cgcgcccgct gccgccgggc cctgcgccgc ttcttcgccc gcgggccgcc cgcgctcacc 240
cacgcactgc tcttctgccc gtgcgcgggc cccgcgtgcg ccgagcgtcg gcgccagacc 300
ttcgtgccct cctgcgcctt ttcggggccc ggccccgcgc cgccctcctg ccttgagccc 360
ttaaacttct gcgagcgcag ccgggtctgc aggtgcgcgc gggcggcggc ggggccgtgg 420
cgagggtggg gacggggcct ctctccggct caccgccctc ccgccgcgca ggcctcgcct 480

cctggccttt caggtctcgt gcaccccagc gcccagcgcc cccgacggct gcctgctgga 540
ccagggcgcc cgctgcctgc gcgcctacgc gggcctcgtg gggtccccgc aggcaccgcc 600
gtcaccccta actacgtgga caacgtgagc gcgcgcgtgg cgccctggtg cgactgcgga 660
gccagcggga accggcgtga ggactgcgaa gccttccggg ggctctttac caggaaccgc 720
tgcttggatg gtgccattca ggcctttgcc agcgggtggc ccccagtcct gctggaccag 780
ctgaaccccc agggagaccc ggagcacagc ctcctgcagg tgtcctccac aggcagggcc 840
ctggagagac gctccctgct ctccatactt cctgtcctgg ctctcccggc cctgctctga 900

<210> 12
<211> 549
<212> DNA
<213> Homo sapiens

<220>
<223> Splice form c encoding putative secreted protein

<400> 12
atggtccgct gcctggggcc tgcgctgctg ctgctgctgt tactggggtc ggcgagctcg 60
gtcggaggga accgatgtgt ggacgcggcc gaagcctgca cggcggacgc gcggtgccag 120
cgtttgcgct ccgagtatgt ggcgcagtgc ctgggccggg ctgcgcaggg gggctgtccc 180
cgcgcccgct gccgccgggc cctgcgccgc ttcttcgccc gcgggccgcc cgcgctcacc 240
cacgcactgc tcttctgccc gtgcgcgggc cccgcgtgcg ccgagcgtcg gcgccagacc 300
ttcgtgccct cctgcgcctt ttcggggccc ggccccgcgc cgccctcctg ccttgagccc 360
ttaaacttct gcgagcgcag ccgggtctgc aggcctcgcc tcctggcctt tcaggtctcg 420
tgcaccccag cgcccagcgc ccccgacggc tgcctgctgg accagggcgc ccgctgcctg 480
cgcgcctacg cgggcctcgt ggggtccccg caggcaccgc cgtcacccct aactacgtgg 540
acaacgtga 549

<210> 13
<211> 2047
<212> DNA
<213> Homo sapiens

<220>
<221> intron
<222> (97)
<223> A genomic sequence containing an approximately 500
bp long intron between positions 97 and 98 (not
shown)

<400> 13
ctatcagact agggctctgc cagccatcct tctctgttga aggtccagca tggtccgctg 60
cctggggcct gcgctgctgc tgctgctgtt actgggtagg gtcggcgagc tcggtcggag 120
ggaaccgatg tgtggacgcg gccgaagcct gcacggcgga cgcgcggtgc cagcgtttgc 180
gctccgagta tgtggcgcag tgcctgggcc gggctgcgca ggggggctgt ccccgcgccc 240
gctgccgccg ggccctgcgc cgcttcttcg cccgcgggcc gcccgcgctc acccacgcac 300
tgctcttctg cccgtgcgcg ggccccgcgt gcgccgagcg tcggcgccag accttcgtgc 360
cctcctgcgc cttttcgggg cccggccccg cgccgccctc ctgccttgag cccttaaact 420

tctgcgagcg cagccgggtc tgcaggtgcg cgcgggcggc ggcggggccg tggcgagggt 480
ggggacgggg cctctctccg gctcaccgcc ctcccgccgc gcaggcctcg cctcctggcc 540
tttcaggtct cgtgcacccc agcgcccagc gcccccgacg gctgcctgct ggaccagggc 600
gcccgctgcc tgcgcgccta cgcgggcctc gtgggtacgc gcggccggga tccgggcgag 660
ggcgggggtt ctccagggga tatctccgcc cgggtgggcc gatgacttcg ccctcagggt 720
ccccgcaggc accgccgtca cccctaacta cgtggacaac gtgagcgcgc gcgtggcgcc 780
ctggtgcgac tgcggagcca gcgggaaccg gcgtgaggac tgcgaagcct tccgggggct 840
ctttaccagg aaccgctgct tgggtgaggg gcccgggggg gagtggaggg ggagtggggg 900
cggcgcttac tgccccctcc caagccgcct ggctgggagc cattttagag gggagaatgg 960
aagactgtac agttgagtca ctctgtcaca gctgtgctta ttgttttgtt attcctcacc 1020
acacaccttc tgtccaagga gccagtcttt gcagcagggg gtctctcact ttgtcccctg 1080
tgctgagccc tgtgctaggg tttcccagct aagtccaccc tggacccctc cctccataga 1140
tggtgccatt caggcctttg ccagcgggtg gcccccagtc ctgctggacc agctgaaccc 1200
ccagggagac ccggagcaca gcctcctgca ggtaggtgca gggaggggag ggtgagctgg 1260
cacctccccc actgtcacct tcacaccctt ccgtccctgg tgggcctggg tggaggcatg 1320
aagggcctgg ggtgggggtg caggcagagg gcagagacag gcttttgcct caagtctgca 1380
cttggctccc acccccaagg tgtcctccac aggcagggcc ctggagagac gctccctgct 1440
ctccatactt cctgtcctgg ctctcccggc cctgctctga ttaggacagc gacctcggat 1500
agcacagcca gctactccac cctgcctgcc tgggccgcct ctgtggccta ctggcccctt 1560
gagaagggac tggcttaccc cccaagccgg ccctggtgct ttcactccgc tgcccctttgt 1620
aggtttggac accctgtgtg ccgtcccctg gggcaaggga tgtaggctgg ggcctgactg 1680
taaagccccc gtctccctgt caggaggcat cttggttgta agtcccttta ttcacagacc 1740
ttgagaccac tggggtctcc cacaaggtgg ggtcaggaga ggtcactttt gtagctgaga 1800
cctctctgga gacccagatc ccctagagca ggtcagagac atccagaatc ccagaattct 1860
aggaaattgt atcagcctcc caagcatata acccccctaag gaatccatcg gacaagaccc 1920
gtctaacact gcatcctccc aactggggca ttacccacca ttgtagccac ctgtgcacca 1980
tgacatgctg ggcagagtct ttcctgttcc ccatatgctg cctgtggagg aaaacctgca 2040
aggggca 2047

<210> 14
<211> 24
<212> PRT
<213> Mus musculus

<220>
<223> Putative signaling sequence from alternative exon
1B

<400> 14
Met Leu Arg Arg Ala His Leu Met Asp Glu Arg Pro Gly Gln Ala Ile
1 5 10 15

Phe Leu Gly Leu Gly Ser Gln Arg
20

<210> 15
<211> 53

<212> PRT
<213> Mus musculus

<220>
<223> Unique region of the secreted isoform (a3/4)

<400> 15
Cys Val Arg Ala Gly Arg Ala Gly Pro Leu Thr Arg Val Arg Ala Arg
1 5 10 15

Ala Gly Pro Val Ser Leu Pro Ser Arg Pro His Ala Leu Pro Arg Pro
20 25 30

Ala Pro Ala Thr Ala Ala Arg Arg Arg Gly Ala Arg Val Val Cys Ala
35 40 45

Ser Thr Gln Ala Ser
50

<210> 16
<211> 49
<212> PRT
<213> Mus musculus

<220>
<223> Putative transmembrane anchor sequence

<400> 16
Ala Arg His Glu Trp Pro Glu Lys Ser Trp Arg Gln Lys Gln Ser Leu
1 5 10 15

Phe Cys Pro Asn Ala Gln Gly Val Leu Ala Val Cys Thr His Cys Pro
20 25 30

Gly Ser Pro Gly Pro Ala Leu Ile Arg Asn Met Asn Arg Gly Arg His
35 40 45

Ser

<210> 17
<211> 16
<212> PRT
<213> Mus musculus

<220>

<223> GPI anchor

<400> 17
Val Ser Trp Leu Tyr Ala Leu Thr Ala Leu Ala Leu Gln Ala Leu Leu
1 5 10 15

<210> 18
<211> 23
<212> DNA
<213> Mus musculus

<220>
<223> 5' RACE primer 1

<400> 18
ttcagctcag tgagcagtca tcg 23

<210> 19
<211> 20
<212> DNA
<213> Mus musculus

<220>
<223> 5' RACE primer 2

<400> 19
caggttgtcc aggtagttgg 20

<210> 20
<211> 20
<212> DNA
<213> Mus musculus

<220>
<223> 5' RACE primer 3

<400> 20
catcgtgcca cgtactcaga 20

<210> 21
<211> 24
<212> DNA
<213> Mus musculus

<220>
<223> 3' RACE primer 1

<400> 21
tacaagcctt tgacagcttg cagc 24

<210> 22
<211> 23
<212> DNA
<213> Mus musculus

<220>
<223> 3' RACE primer 2

<400> 22
agagctggag gcagaaacag tcc 23

<210> 23
<211> 25
<212> DNA
<213> Mus musculus

<220>
<223> Exon 1a sense primer 1

<400> 23
ccaccatggc ccactgcatg gagtc 25

<210> 24
<211> 20
<212> DNA
<213> Mus musculus

<220>
<223> Exon 1b sense primer 2

<400> 24
ccaccatgtt gagaagagca 20

<210> 25
<211> 20
<212> DNA
<213> Mus musculus

<220>
<223> Exon 2 sense primer 3

<400> 25
gtgtacagca gacgagcggt 20

<210> 26
<211> 22
<212> DNA
<213> Mus musculus

<220>
<223> Exon 5 sense primer 5

<400> 26
atacaagcct ttgacagctt gc 22

<210> 27
<211> 21
<212> DNA
<213> Mus musculus

<220>
<223> Exon 4 antisense primer 4

<400> 27
gttccttgta aagagcttgc g 21

<210> 28
<211> 21
<212> DNA
<213> Mus musculus

<220>
<223> Exon 6 antisense primer 6

<400> 28
tggacaagat gcctactgac g 21

<210> 29
<211> 49
<212> DNA
<213> Homo sapiens

<220>
<223> Exon1 (5' UTR)

<400> 29
ctatcagact agggctctgc cagccatcct tctctgttga aggtccagc 49

<210> 30
<211> 46
<212> DNA
<213> Homo sapiens

<220>
<223> Exon1 (translated region)

<400> 30
atggtccgct gcctggggcc tgcgctgctg ctgctgctgt tactgg 46

<210> 31
<211> 346
<212> DNA
<213> Homo sapiens

<220>
<223> Exon2

<400> 31
ggtcggcgag ctcggtcgga gggaaccgat gtgtggacgc ggccgaagcc tgcacggcgg 60
acgcgcggtg ccagcgtttg cgctccgagt atgtggcgca gtgcctgggc cgggctgcgc 120
agggggggctg tccccgcgcc cgctgccgcc gggccctgcg ccgcttcttc gcccgcgggc 180
cgcccgcgct cacccacgca ctgctcttct gcccgtgcgc gggccccgcg tgcgccgagc 240
gtcggcgcca gaccttcgtg ccctcctgcg ccttttcggg gcccggcccc gcgccgccct 300
cctgccttga gcccttaaac ttctgcgagc gcagccgggt ctgcag 346

<210> 32
<211> 79
<212> DNA
<213> Homo sapiens

<220>
<223> Intron 2

<400> 32
gtgcgcgcgg gcggcggcag ggccgtggcg agggtgggga cggggccttt ctccgggtca 60
ccgccctccc gccgcgcag 79

<210> 33
<211> 110
<212> DNA
<213> Homo sapiens

<220>
<223> Exon3

<400> 33
gcctcgcctc ctggcctttc aggtctcgtg caccccagcg cccagcgccc ccgacggctg 60
cctgctggac cagggcgccc gctgcctgcg cgcctacgcg ggcctcgtgg 110

<210> 34

<211> 94
<212> DNA
<213> Homo sapiens

<220>
<223> Intron3 (83 bp for alternative spliced putative
secreted form)

<400> 34
gtacgctcgg ccgggatccg gccgagggcg ggggttctcc aggggatata tccgcccggg 60
tgggccgatg acttcgccct cagggtcccc gcag 94

<210> 35
<211> 135
<212> DNA
<213> Homo sapiens

<220>
<223> Exon4

<400> 35
gcaccgccgt cacccctaac tacgtggaca acgtgagcgc gcgcgtggcg ccctggtgcg 60
actgcggagc cagcgggaac cggcgtgagg actgcgaagc cttccggggg ctctttacca 120
ggaaccgctg cttgg 135

<210> 36
<211> 276
<212> DNA
<213> Homo sapiens

<220>
<223> Intron4

<400> 36
gtgaggggcc cgggggggag tggaggggga gtgggggcgg cgcttactgc cccctcccaa 60
gccgcctggc tgggagccat tttagagggg agaatggaag actgtacagt tgagtcactc 120
tgtcacagct gtgcttattg ttttgttatt cctcaccaca caccttctgt ccaaggagcc 180
agtctttgca gcaggggtc tctcactttg tcccctgtgc tgagccctgt gctagggttt 240
cccagctaag tccaccctgg acccctccct ccatag 276

<210> 37
<211> 92
<212> DNA
<213> Homo sapiens

<220>
<223> Exon5

<400> 37
atggtgccat tcaggccttt gccagcgggt ggcccccagt cctgctggac cagctgaacc 60
cccagggaga cccggagcac agcctcctgc ag 92

<210> 38
<211> 168
<212> DNA
<213> Homo sapiens

<220>
<223> Intron5

<400> 38
gtaggtgcag ggaggggagg gtgagctggc acctccccca ctgtcacctt cacacccttc 60
cgtccctggt gggcctgggt ggaggcatga agggcctggg gtgggggtgc aggcagaggg 120
cagagacagg cttttgcctc aagtctgcac ttggctccca cccccaag 168

<210> 39
<211> 81
<212> DNA
<213> Homo sapiens

<220>
<223> Exon6 (translated region)

<400> 39
gtgtcctcca caggcagggc cctggagaga cgctccctgc tctccatact tcctgtcctg 60
gctctcccgg ccctgctctg a 81

<210> 40
<211> 557
<212> DNA
<213> Homo sapiens

<220>
<223> Exon6 (3' UTR)

<400> 40
ttaggacagc gacctcggat agcacagcca gctactccac cctgcctgcc tgggccgcct 60
ctgtggccta ctggcccctt gagaagggac tggcttaccc cccaagccgg ccctggtgct 120
ttcactccgc tgccctttgt aggtttggac accctgtgtg ccgtcccctg gggcaaggga 180
tgtaggctgg ggcctgactg taaagccccc gtctccctgt caggaggcat cttggttgta 240
agtcccttta ttcacagacc ttgagaccac tggggtctcc cacaaggtgg ggtcaggaga 300
ggtcactttt gtagctgaga cctctctgga gacccagatc ccctagagca ggtcagagac 360
atccagaatc ccagaattct aggaaattgt atcagcctcc caagcatata acccctaag 420
gaatccatcg gacaagaccc gtctaacact gcatcctccc aactggggca ttacccacca 480
ttgtagccac ctgtgcacca tgacatgctg ggcagagtct ttcctgttcc ccatatgctg 540
cctgtggagg aaaacct 557

<210> 41
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: -

<400> 41
cacgttgtcc aggtagttgg 20

<210> 42
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: -

<400> 42
gcactgcgcc acatactcgg a 21

<210> 43
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: -

<400> 43
gctccgagta tgtggcgcag t 21

<210> 44
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: -

<400> 44
gctcacccac gcactgctct tctg 24

<210> 45
<211> 24

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: -

<400> 45
cctaactacg tggacaacgt gagc 24

<210> 46
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: -

<400> 46
atggtgccat tcaggccttt gccag 25

<210> 47
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: -

<400> 47
gcaggtgtcc tccacaggca g 21

<210> 48
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: -

<400> 48
gaagtatgga gagcagggag cgtc 24

<210> 49
<211> 21
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: -

<400> 49
ccaccatggt ccgctgcctg g 21

<210> 50
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: -

<400> 50
gaggtcgctg tcctaatcag ag 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 00/00994

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 14/71, A61K 38/17, C12N 5/16
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, A61K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Nature, Volume 398, March 1999, Teresa M. Gunn et al, "The mouse mahogany locus encodes a transmembrane form of human attractin", page 152 - page 156, page 153, right column	1-15
A	--	16-18
X	Current Biology, Volume 8, 1998, Yasushi Enokido et al, "GFRalpha-4 and the tyrosine kinase Ret form a functional receptor complex for persephin" page 1019 - page 1022	1-15
A	--	16-18

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 May 2001	03 -07- 2001
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz/BS Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 00/00994

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Neuro Report, Volume 9, 1998, B. K. Dey et al, "Cloning of a novel murine isoform of the glial cell line-derived neurotrophic factor receptor" page 37 - page 42	1-15
A	--	16-18
E,X	WO 0102557 A1 (JANSSEN PHARMACEUTICA N.V.), 11 January 2001 (11.01.01), page 4, lines 26-30	1-15
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P,X	Molecular and Cellular Neuroscience, Volume 15, 2000, Maria Lindahl et al, "Expression and Alternative Splicing of Mouse Gfra4 Suggest Roles in Endocrine Cell Development" page 522 - page 533	1-15
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P,X	The Journal of Biological Chemistry, Volume 275, No 50, December 2000, Stefan Masure et al, "Mammalian GFRalpha-4, a Divergent Member of the GFRalpha Family of Coreceptors for Glial Cell Line-derived Neurotrophic Factor Family Ligands, Is a Receptor for the Neurotrophic Factor Persephin", page 39427 - page 39434	1-15
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A	WO 9950298 A1 (MILLENNIUM PHARMACEUTICALS, INC.), 7 October 1999 (07.10.99), page 8, lines 3-15	1-18
	--	
X	WO 9962332 A1 (AIRAKSINEN, MATTI), 9 December 1999 (09.12.99)	16-18
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P,X	EMBL/GenBank/DDBJ databases, Accession no. AL 356755, Sycamore N.:"Human DNA sequence from clone RP5-964F7 on chromosome 20 Contains part of a gene for a putative GDNF family receptor alpha 4 protein, the 3' part of a gene for a novel protein (disintegrin and metalloproteinase), ESTs, STSs, GSSs and CpG islands",23 May 2000	1-15
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 00/00994

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EMBL/GenBank/DDBJ databases, Accession no. AF253318, Zhou B. et al: "Homo sapiens GFR receptor alpha 4 protein (GFRA4) mRNA, complete cds.", 26 October 2000 --	1-15
A	EMBL/GenBank/DDBJ databases, Accession no. AC17113, Waterston R.H: "Homo sapiens chromosome 20 clone RP11-574H7, Working Draft Sequence, 31 unordered pieces", 14 December 1999 --	1,3-9,11-15
A	EMBL/GenBank/DDBJ databases, Accession no. AC013324, Birren B. et al: "Homo sapiens chromosome 2 clone RP11-388K24 map 2, Low-pass sequence sampling", 9 November 1999 --	1,3-9,11-15
A	EMBL/GenBank/DDBJ databases, Accession no. AP002898, Yada T. et al: "Homo sapiens genomic DNA, chromosome 20 p, clone: 13N6, 25 October 2000 -- --------	1,3-9,11-15

INTERNATIONAL SEARCH REPORT

Int nal application No.
PCT/FI00/00994

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 18
because they relate to subject matter not required to be searched by this Authority, namely:

see next sheet*

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet**

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

*
Claim 18 relates to methods of treatment of the human or animal body by surgery or by therapy as well as diagnostic methods (Rule 39.1(iv). Nevertheless, a search has been executed for this claim. The search has been based on the alleged effects of the compounds/compositions.

**

Unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features"-i.e. features that define a contribution which each of the inventions makes over the prior art (c.f. PCT Rule 13.2).

In view of the prior art in the present case such a unifying, novel technical feature has not been found between the different inventions revealed in the claims.

The prior art is represented by Gunn TM et al., Nature, Vol 398, 11 March 1999, pages 152-156, which discloses the localization of the mouse GFRα4 gene. Thus, a mammalian GFRα4 is known in the prior art and therefore it is considered to be obvious to a person skilled in the art to determine the cDNA for the receptor. No special technical feature has been shown to be present between the different sequences claimed. In the description to the present application it is stated that the mouse and human GFRα4 lack the first Cys-rich domain common for all known GFRα4-receptors (page 7, first paragraph). However, this discovery has not been shown to lead to any industrial application involving a unifying inventive concept.

Therefore, the international searching authority has arrived at the following principle of division:

Invention A, claims 1 (partly), 2, 4 (partly), 6-9 (partly), 10, 12-15 (partly), pertains to murine GFRα4 corresponding to the sequences SEQ ID NOs: 1-3, 7-9.

Invention B, claims 1 (partly), 3, 4 (partly), 5, 6-9 (partly), 11, 12-15 (partly) pertains to human GFRα4 corresponding to the sequences SEQ ID Nos: 4-6, 10-13, 29-39.

.../...

Invention C, claims 16-18, concerns a method for obtaining a cell-line useful in the production of a transgenic non-human animal. The method involves inactivating a functional GFRα4 receptor. The method cannot be seen to involve any unifying special technical feature in common with mouse or human GFRα4.

INTERNATIONAL SEARCH REPORT

Information on patent family members

28/05/01

International application No.
PCT/FI 00/00994

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0102557 A1	11/01/01	AU 5810200 A	22/01/01
		GB 9915200 D	00/00/00
WO 9950298 A1	07/10/99	AU 3206899 A	18/10/99
WO 9962332 A1	09/12/99	AU 4618299 A	20/12/99
		FI 981217 A	30/11/99